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CONTENTS TABLE DES MATIÈRES INHALT

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ON THE NATURE OF P.P.L.O.
I. BACTERIOLOGICAL OBSERVATIONS

by

A. CHARLOTTE RUYS
with the technical assistance of N. E. Edel

(Received October 1, 1959).

I. Introduction.

In a previous communication on the serology of *Pleuropneumonia*-like organisms (P.P.L.O.) with HUYSMANS-EVERS (1956) the value of growth inhibition for the identification with specific sera was shown on a number of strains of human origin (*M. hominis, M. fermentans, M. salivarium*) and two bovine strains (*M. mycoides*). In these experiments cross reactions were not observed.

However, difficulties were encountered with two genital strains, which biochemically seemed to belong to *M. hominis* and which initially showed inhibition with specific serum. Later, however, no growth inhibition could be observed with any of our mycoplasma-sera. These two strains then possessed a somewhat coarse and granular colony appearance and could be cultured on media without enrichment with serum or ascitic fluid. Further studies with these strains were dropped for some time but they were kept in subculture on the P.P.L.O. medium, both with and without enrichment with serum.

All our P.P.L.O. strains are kept in the freeze-dried state, many from 1953/54 on, whereas some were also subcultured fortnightly.

When resuming the studies of the P.P.L.O. strains in 1957 we experienced more and more difficulties, because several strains which previously had given clear cut results with their specific sera, after a varying number of subcultures could no more be inhibited, although the sera used still proved active in other strains of the same species.

When making smears of these non-reactive strains we were surprised to find minute coccoid elements which were either Gram positive or Gram variable (Fig. 1). This finding made us check the other strains of our collection too.

On examination part of our strains turned out to be still pure cultures of P.P.L.O. whereas others contained besides P.P.L.O. coccoid elements. Of some strains we possessed a line of pure P.P.L.O. besides a mixed one. In order to ascertain whether these coccoid elements were an outside contamination or could in some way belong to the P.P.L.O. the following studies were made.

II. Methods.

a. Medium. We used the P.P.L.O. medium of Klieneberger in a slightly modified form (see Huysmans-Evers and Ruys, 1956), generally enriched either with 30% horse serum or ascitic fluid. The final agar-concentration was 1.75%.

The cultures were incubated at 37° C. for 4 days either in a McIntosh anaerobic jar or aerobically, after which the plates were kept at room temperature.

b. Examination of colonies. The appearance of the colonies was studied microscopically by low power magnification (160×) with a phase contrast microscope, either directly on the agar medium or in vertical cuts through the colonies. The latter preparations were made by cutting with a razor blade ca 2 mm thick vertical sections. The unfixed preparations were immersed in 70% alcohol with a few drops of methylene blue-azur II-solution [1]) and were studied in this solution on slides under sealed coverslips.

c. Serology. The sera were prepared in rabbits, by intravenous inoculation of washed (3×) suspensions of P.P.L.O. grown in P.P.L.O. media.

Growth inhibition was tested by putting disks of filter paper (7 mm) soaked in serum in the middle of a streak of a broth culture inoculated on the solid P.P.L.O. medium.

The agglutination experiments were made with a suspension in saline of coccoid elements in pure culture. Generally one drop of serum on a slide was mixed with one drop of the suspension. After rocking for a few minutes the results were read with a hand lens.

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[1]) Prescription of A. van den Hooff for the examination of L colonies

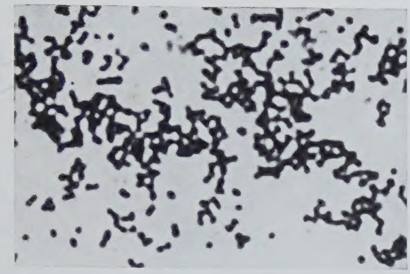

Fig. 1.

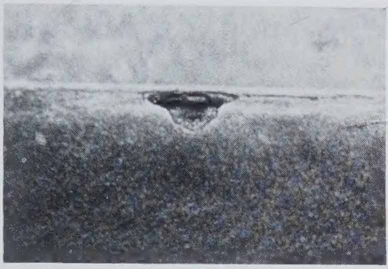

Fig. 2.

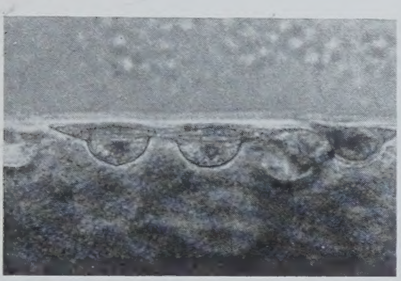

Fig. 3.

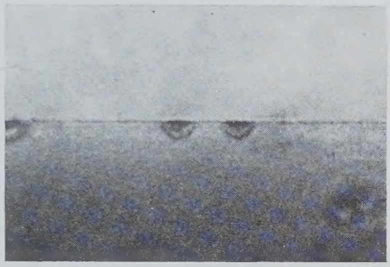

Fig. 4.

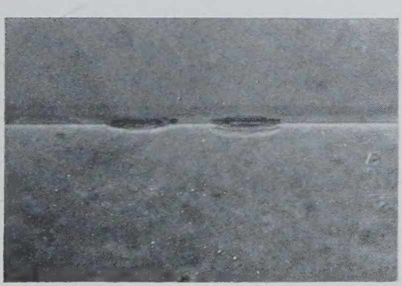

Fig. 5.

Fig. 6.

Fig. 1. Gram stained smear of coccoid elements of *Mycoplasma salivarium* (K 8). 2000 ×.

Fig. 2–6. Vertical cuts through colonies of *Mycoplasma* and coccoid elements grown on P.P.L.O. medium for 4 days at 37° C. Stained with methylene blue-azur II in 70 % alcohol. Phase contrast microscope 175 ×.

Fig. 2. *Mycoplasma salivarium* (K 23). P.P.L.O. colony only. 175 ×.
Fig. 3. *Mycoplasma salivarium* (K 18). P.P.L.O. colonies only. 175 ×.
Fig. 4. *Mycoplasma fermentans* (V 58). P.P.L.O. colonies only. 175 ×.
Fig. 5. *Mycoplasma fermentans* (V 58). Colonies of coccoid elements. 175×.
Fig. 6. *Mycoplasma hominis* (V 32). P.P.L.O. colony and colony of coccoid elements. 175 ×.

In those cases in which the suspension of the cocci was insufficiently thick a sample of the culture was rubbed directly into the specific serum and into the control rabbit serum.

III. **Results.**

a. Colony appearance.

1. *Mycoplasma* strains in which in Gram stained smears no coccoid elements were observed showed on direct microscopical examination colonies of varying size. The smaller ones of these colonies appear of a fairly homogeneous structure, the larger ones have a sharply edged central part surrounded by a brim (fig. 7) as described for P.P.L.O. by many authors.

The vertical sections demonstrate that these colonies all grow into the agar, those with a brim having the shape of a hat (fig. 2, 3, 4).

2. In the lines of subcultures containing the coccoid elements colonies were encountered of a size comparable to the largest pure P.P.L.O. colonies but of a much coarser appearance (fig. 7). In vertical sections it is seen that these colonies do not penetrate into the medium, their development being restricted to a sharply delimited flat-lens shape at the agar surface (fig. 5). In the mixed cultures the hat-like colonies which penetrate the agar are also present (fig. 6).

b. Properties of coccoid elements.

We succeeded in obtaining pure cultures of coccoid elements by growing the mixed strains on the P.P.L.O. medium without serum or ascitic fluid. On this poor medium the P.P.L.O. forms are suppressed and they do not reappear when the strain is propagated again on the enriched medium. The latter observation is based on the absence of agar penetrating colonies in vertical sections.

1. Morphology. The tiny coccoid elements, often occurring in clussters, measure in Gram-stained smears 0.5 μ or less. In old cultures of these elements smaller and larger forms may appear. Generally these bodies are Gram positive or Gram variable, but they become Gram negative on prolonged decolorization.

2. Nutritional requirements and biochemistry.

The coccoid elements are easily maintained on not enriched P.P.L.O. medium. Their development under aerobic conditions is better than

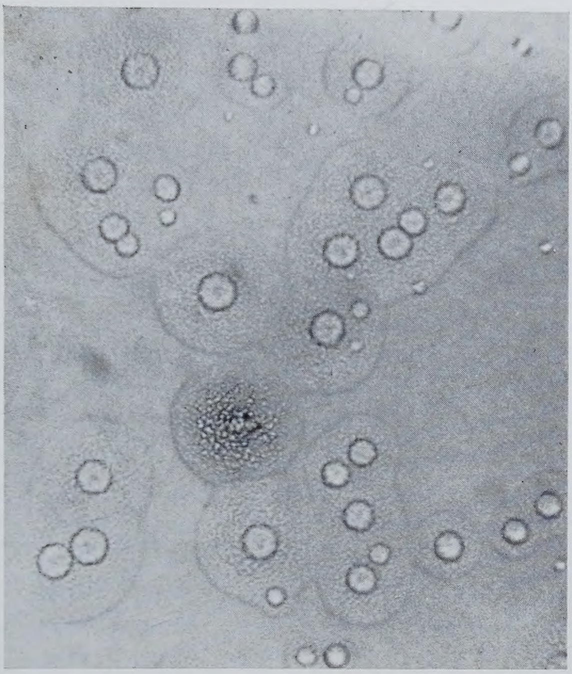

Fig. 7. *Mycoplasma fermentans* (V 58) on P.P.L.O. medium after 4 days at 37° C. In center colony of coccoid elements. 110 ×.

that of the P.P.L.O. from which they were derived. In P.P.L.O. broth the coccoid elements grow scantly, with even less turbidity than cultures of P.P.L.O. On ordinary nutrient agar growth is only slight.

We could not demonstrate any fermentation of sugars by coccoid elements. Whereas *M. fermentans* in P.P.L.O. broth with 1% glucose or mannitol and neutral red showed acid formation in 3 days, its pure line of coccoid elements proved inactive.

The coccoid elements are catalase negative. Horseblood was not attacked by the coccoid elements, but the P.P.L.O. strains from which they were derived had lost this property on subculturing too. These elements are sensitive to penicillin up to 0.5 U/ml. They are not inhibited by thallium acetate (0.125 g/l).

3. Serology. Growth inhibition. The growth of pure strains of coccoid elements was not inhibited by the sera prepared with their corresponding P.P.L.O. strains. Comparably to what is known from ordinary bacteria the coccoid elements were not inhibi-

ted by sera of rabbits immunized with pure cultures of coccoid elements.

However, once a serum was obtained prepared with a pure culture of coccoid elements from *M. fermentans* which inhibited the growth of two *M. fermentans*-strains but no *M. hominis* or *M. salivarium*-strains.

Agglutination. All the sera prepared with P.P.L.O. elements – also of lines which never carried coccoid elements (G, K19, *M. mycoides* A) – agglutinated the pure coccus lines isolated from human strains. As a control sera were used of normal rabbits and of rabbits immunized with streptococci or meningococci. All controls gave negative results (Table 1).

TABLE 1.
Agglutination of coccoid elements with sera active against *Mycoplasma* strains.

coccoid elements of	rabbit sera prepared with							
	M. ferm. V26	*M. ferm.* G	*M. hom.* V32	*M. saliv.* K19	*M. myc.* A	Contr.	*Strepto-coccus*	*Menin-gococcus*
M. fermentans V26	+	+	+	+	+	—	—	—
M. hominis V32	+	+	+	+	+	—	—	—
M. hominis V45	+	+	+	+	+	—	—	—
M. salivarium K4	+	+	+	+	+	—	—	—
M. salivarium K8	+	+	+	+	+	—	—	—

Rabbit sera prepared with coccoid elements of *M. fermentans* (V58) and with *M. hominis* (V45) equally agglutinated the coccoid elements from all the investigated strains of the three human *Mycoplasma*-species.

IV. Discussion.

The coccoid elements isolated from a number of *Mycoplasma* cultures are defined by the following characteristics. The minute elements are in Gram stained smears ca 0.4 – 0.5 μ in diameter. They are Gram positive or Gram variable. They grow aerobically and anaerobically on nutrient media, which need not be enriched with serum or ascitic fluid, in very small colonies of generally ca 0.1 mm in diameter. In nutrient broth they give a poor growth without

visible turbidity or sediment. They are catalase negative and they do not ferment sugars. They are sensitive to penicillin (0.5 U/ml). They are not inhibited by thallium acetate (0.125 g/l).

As will be discussed in the next communications on studies with the electron microscope (W. VAN ITERSON and A. CH. RUYS) the coccoid elements are of definite bacterial structure in view of their typical cell wall and nuclear region surrounded by cytoplasm. However, we have not succeeded in finding in BERGEY'S Manual or elsewhere the description of such minute cocci of similar characteristics.

The following arguments make an outside contamination improbable and are therefore in favour of the relationship.

1. Of the 26 strains of our collection some proved to be mixed with coccoid elements already in 1954 at the time of freeze drying. Others, which were subcultured fortnightly remained free of coccoid elements up to the present.

However, a number of strains started to carry coccoid elements on occasions separated by a considerable length of time, whereas other strains propagated on the same batches of media remained free of coccoid elements.

2. We never encountered coccoid colonies outside an inoculation streak.

The following observations point to a relationship between the cocci and P.P.L.O.

The fact that sera prepared with pure P.P.L.O. strains in which we could never demonstrate coccoid elements agglutinated the various coccoid strains prompts the conclusion that there exists a serological group relationship between these strains and the coccoid elements. This relationship is corroborated by the observation that we succeeded in preparing a serum with coccoid elements which showed growth inhibition of the P.P.L.O. form of the strain from which it was derived.

A fact which might be considered to be an argument against the relationship is that we have not been able to differentiate the coccoid elements of the three human P.P.L.O. strains from each other by biochemical or serological methods.

Up to now we have not observed a reversion of pure cultures of coccoid elements to P.P.L.O. forms. However, experiments along these lines have been initiated only recently.

The possibility that the occurrence of coccoid elements in cultures of P.P.L.O. is comparable to the relation of L forms and their bacteria will be considered further in the next communication (W. van Iterson and A. Ch. Ruys) which deals with electron microscope studies of these microbes.

Summary.

In a number of strains of the three species of P.P.L.O. of human origin coccoid elements have been observed, which are Gram positive or Gram variable and of very small size ($< 0.5\ \mu$). They are able to grow in pure culture on the surface of nutrient agar in very small colonies. Although they have bacterial characteristics, they could not be identified as known bacteria. Agglutination tests are in favour of a serological relationship between the coccoid elements and P.P.L.O. The suggestion that these elements might be an outside contamination is made unlikely by several observations. The supposition that P.P.L.O.s are L forms of these coccoid elements should be considered further.

References.

Huysmans-Evers, A. G. M. and Ruys, A. Ch. 1956. Microorganisms of the *Pleuropneumonia* group (Family of *Mycoplasmataceae*) in man. Antonie van Leeuwenhoek **22**, 371.

(Laboratory of Electronmicroscopy and Laboratory of Hygiene, University of Amsterdam).

ON THE NATURE OF P.P.L.O.

II. ELECTRON MICROSCOPY

by

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with the technical assistance of Sj. Verduyn Lunel-Fokkema

(Received October 1, 1959).

I. Introduction.

During the 4–6 years of maintenance in the laboratory of 26 strains of *Mycoplasma*, in a number of subcultures Gram positive coccoid elements were observed after various lapses of time. These minute bodies appeared in several strains of the three human species *Mycoplasma hominis*, *M. fermentans* and *M. salivarium*; in the two simultaneously subcultured *M. mycoides* strains and in other strains of the three human species such elements were never detected. In the preceding communication (Ruys, 1960) it was suggested, on the basis of serology and the unlikelihood of contamination, that the P.P.L.O. elements might perhaps represent L forms of minute cocci. The present communication is confined to electron-microscopical information which may lend support to this latter view. In a separate electron-microscopical study (van Iterson and Ruys, 1960) the general morphological features of the human species of the *Mycoplasmataceae* are analysed.

II. Methods.

Electron micrographs were made after application of two different techniques, for the details of which is referred to van Iterson and Ruys (1960). Firstly, the P.P.L.O. elements were studied in their entirety in shadowed specimens. To this end the *Mycoplasmataceae*

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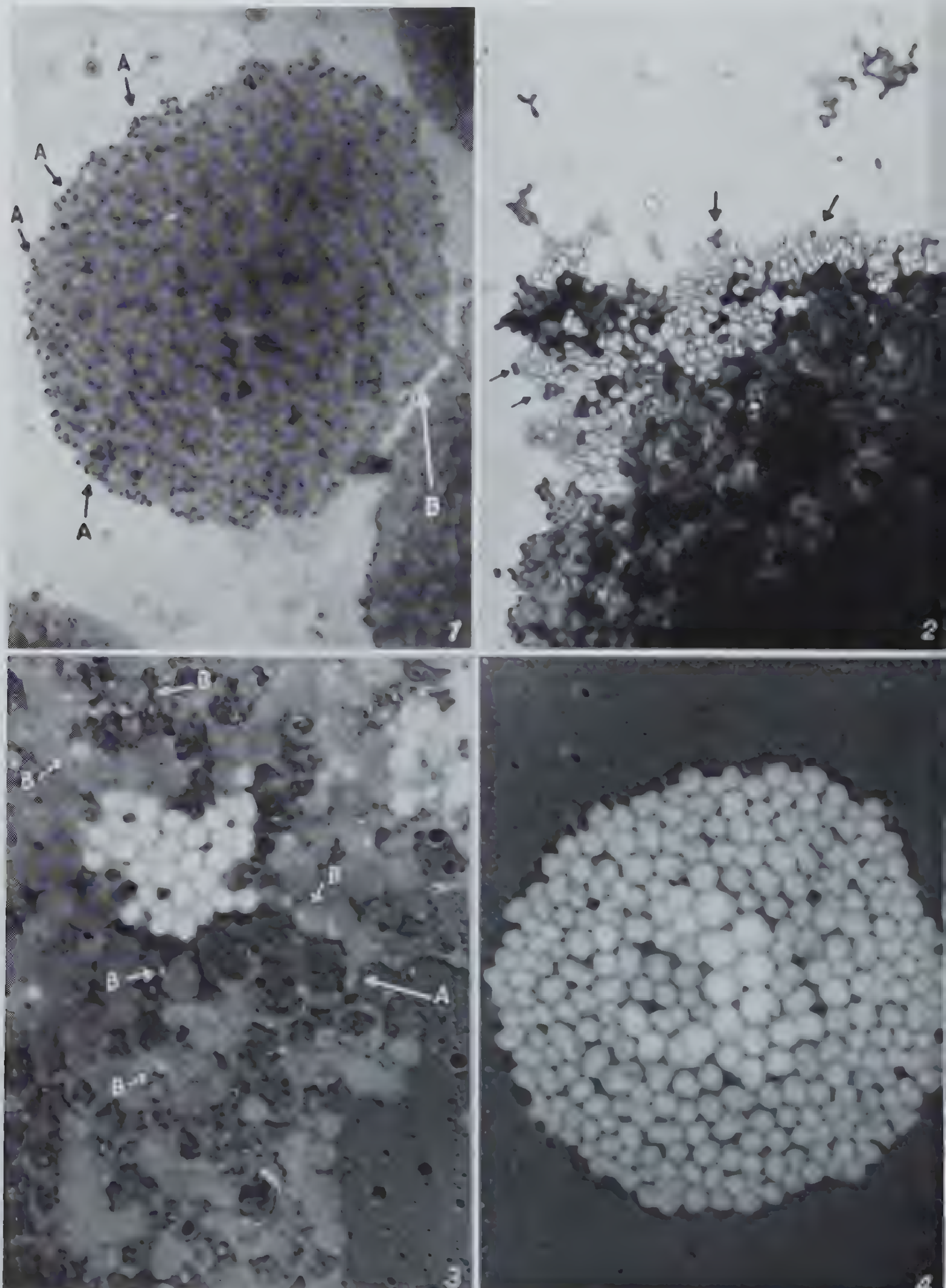
[1]) We gratefully acknowledge the grants received in 1957 and 1958 in support of the present investigation from the Netherlands Organization for Pure Research (Z.W.O.).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were cultivated 0–14 days on collodion membranes spread over the solid culture medium in Petri dishes. Since the clarity of the electron-microscopical image tends to be spoiled by particles from the serum contained in the P.P.L.O. agar, the latter was covered by a minute layer of tryptone agar before making the collodion film (Kellenberger, Liebermeister and Bonifas, 1956). Inoculation was performed by either moving carefully an agar block with a culture over the membrane or by running over it a suspension of a P.P.L.O. culture in salt solution or in a nutrient medium.

The second technique: ultra-microtomy of the subsurface agar colonies was realised by application of the principles of the fixation method of Ryter and Kellenberger (1958) and embedding in methacrylate.

III. **Results.**

The coccoid elements could be demonstrated without difficulties in all strains in which they were expected from the light-microscopical inspection (cf. Ruys, 1960). Conversely: in the strains supposedly free of these elements they were never encountered, with the exception of a case in *Mycoplasma salivarium* to be discussed below. Because electron microscopy requires little material it may perhaps be concluded that the coccoid elements, when present, occur in fair distribution.

The presence of coccoid elements can be discerned in the light micrograph reproduced in fig. 1. The colony is from a mixed culture

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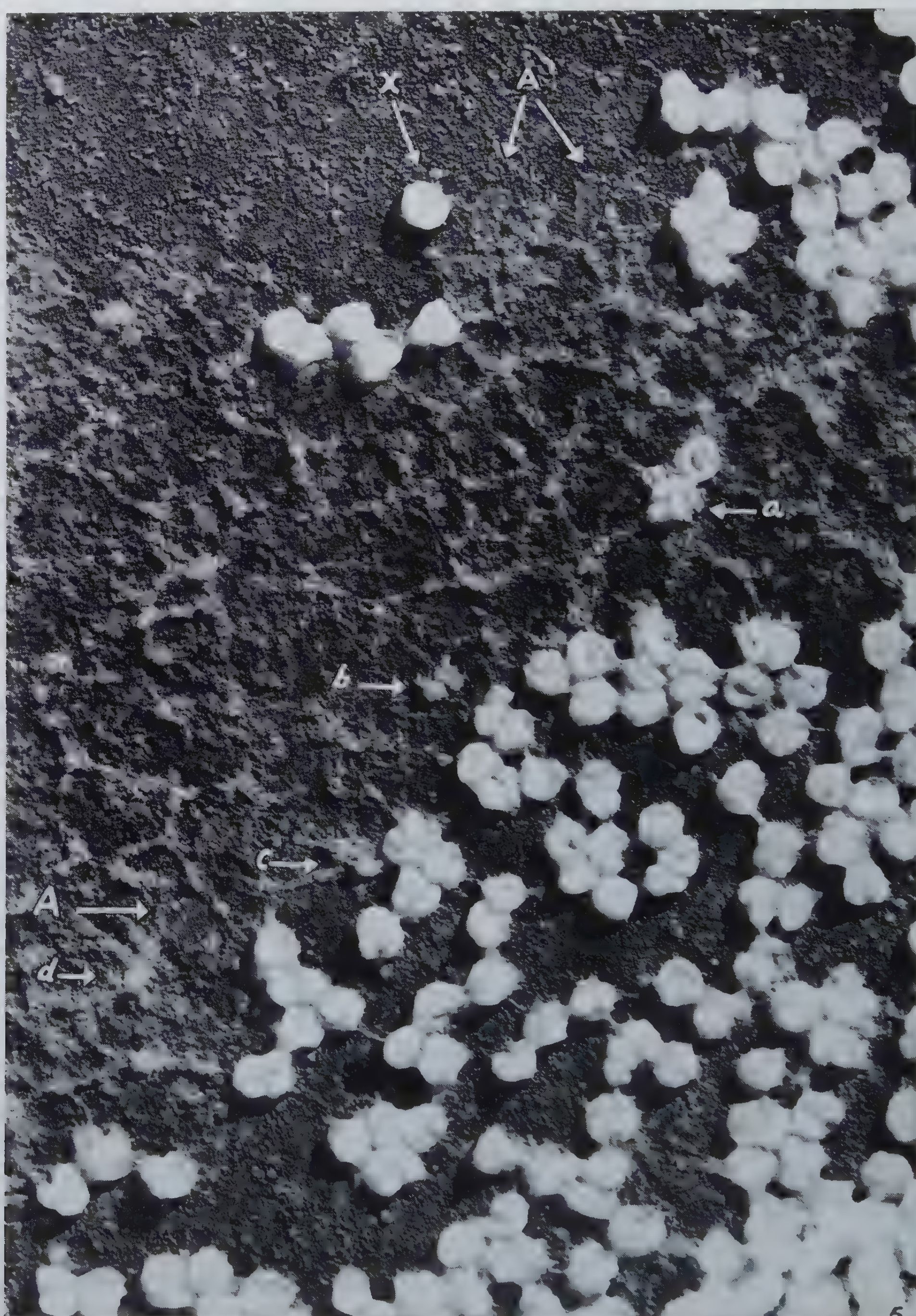
Fig. 1. Light micrograph of an impression preparation on a coverslip of a 10 days' old culture of *M. hominis*. Fixation through the agar in Bouin's solution, staining with Giemsa solution. Note mixture of light vesicles (e.g. at B) and more intensely stained cocci (e.g. at A). × 1,600.

Fig. 2. Electron micrograph of the edge of a 4 days' old colony of *M. hominis* grown on a collodion membrane over P.P.L.O. agar. Note the random distribution of the small cocci in the colony. Inoculation by streaking an agar block with a culture over the membrane. × 1,600.

Fig. 3. Pd shadow; like all following pictures with shadow printed in reverse. A 5 days' old development of *M. hominis*, on the upper surface of the collodion membrane, consisting of flattened vesicles and coccoid elements. At arrow A: empty collapsed vesicles; at B: small dense particles. Inoculation with a block of agar. × 6,250.

Fig. 4. Pure colony of small coccoid elements grown on the upper side of a collodion membrane in a 4 days' old culture of *M. hominis*. Inoculation with an agar block. × 6,250.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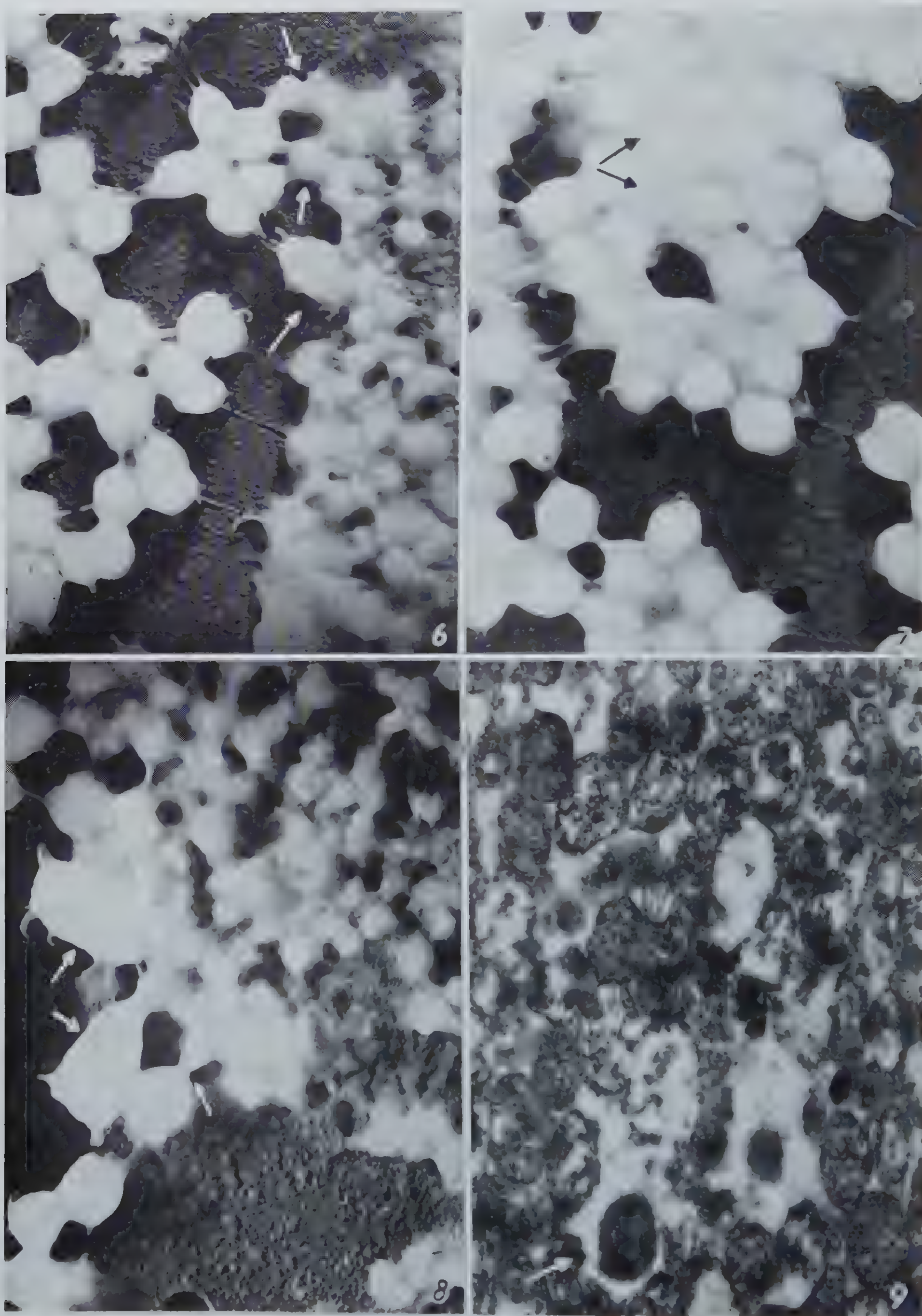
of *Mycoplasma hominis* (strain U6N). The specimen is an impression preparation on a coverslip, Giemsa stained after fixation through the agar with Bouin's solution (cf. KLIENEBERGER-NOBEL, 1950). In the colony the two composing elements can be vaguely distinguished: the cocci as more intensely stained granules (arrows A) and lighter structures (arrow B) which in the shadowed electron micrographs appear as vesicles.

This feature can be studied more easily in an electron micrograph at comparable magnification (fig. 2). In the edge of this 4 days' old colony of *M. hominis* (U6N) the granules appear with much more contrast than the typical P.P.L.O. units. Quite strikingly the coccoid elements in fig. 2 are not confined to one or more separate colonial regions. They are even found singly or in groups of two or more connected to the vesicles (arrows). Generally such an intermingling of the elements can hardly be expected when two different species are developing by chance in the same area. It could, however, be questioned whether or not the distribution is influenced by the way of inoculation *i.e.* by rubbing an agar block with a culture over the collodion film.

In fig. 3 of a 5 days' old culture of the same *M. hominis* cocci are observed in a small colony together with flattened vesicles. At arrows A empty collapsed vesicles with larger diameters can be seen. Dense particles of various sizes can be noticed at arrows B, supposedly in connection with the vesicles.

Pure colonies of cocci can also be found, as illustrated in fig. 4, from a 4 days' old culture again from *M. hominis* U6N. The size of these cocci varies between $\sim 0.7\ \mu$, the central ones, and $\sim 0.45\ \mu$ at the periphery. Several of these cocci, in particular the smaller ones, appear in division. Such a colony on the collodion membrane may be considered as the equivalent of the flattened lens-shaped colonies on agar media. Therefore, the Gram-positive elements of fig. 1 and 5 from the previous communication (RUYS, 1960) are here identified as cocci of unusually small size. As will be discussed below, these cocci possess a true bacterial structure.

Fig. 5. Development encountered on the lower side of the membrane in a 6 days' old culture of *M. fermentans*. It is supposed that the ridges mark outlines of collapsed vesicles. At A: the outer surface of a vesicle membrane can be observed. Note scattered distribution of the cocci. At a, b, c, d: elements that are not quite shaped like cocci but might be related to them. Inoculation with a suspension. × 1,2500.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In our shadowed preparation of *Mycoplasma fermentans* (strains V26 and V58) vesicles can rarely be recognised with the same precision as in *M. hominis* and *M. salivarium*. However, in all thin-sections of adequately fixed material the vesicles proved to be the integrating constituent also of the *M. fermentans* colonies (VAN ITERSON and RUYS, 1960). We therefore interpret the numerous ridges in fig. 5 to mark the outlines of collapsed vesicles. We assume that much of the more solid contents of the vesicles on drying precipitated against the bordering membranes, which is in agreement with our experience on the sectioned material when badly fixed. The vesicles must have been fluid-filled to a surprising extent. The cocci on the other hand stand out as opaque structures with considerable shadows.

Comparably to the picture in fig. 2 the cocci in fig. 5 appear to be distributed randomly. In the present preparation, however, this can with certainty not be attributed to the mode of inoculation. Like in all other preparations the inoculation was performed on the upper surface of the collodion membrane, but this specimen was made by turning the membrane upside down and shadowing its side facing the nutrient agar. The organisms visible in the illustration must therefore originate from elements that passed a few occasional holes in the membrane. As yet it seems impossible to judge whether, for instance, the isolated coccus at arrow X was produced by division of other cocci or whether this organism could in some way have originated from vesicles. An argument in favour of the supposition that the cocci do not necessarily originate from other cocci can be found in the accumulations of dense material at arrows a, b, c and d. The elements at arrow a could perhaps be taken as a coccus dividing

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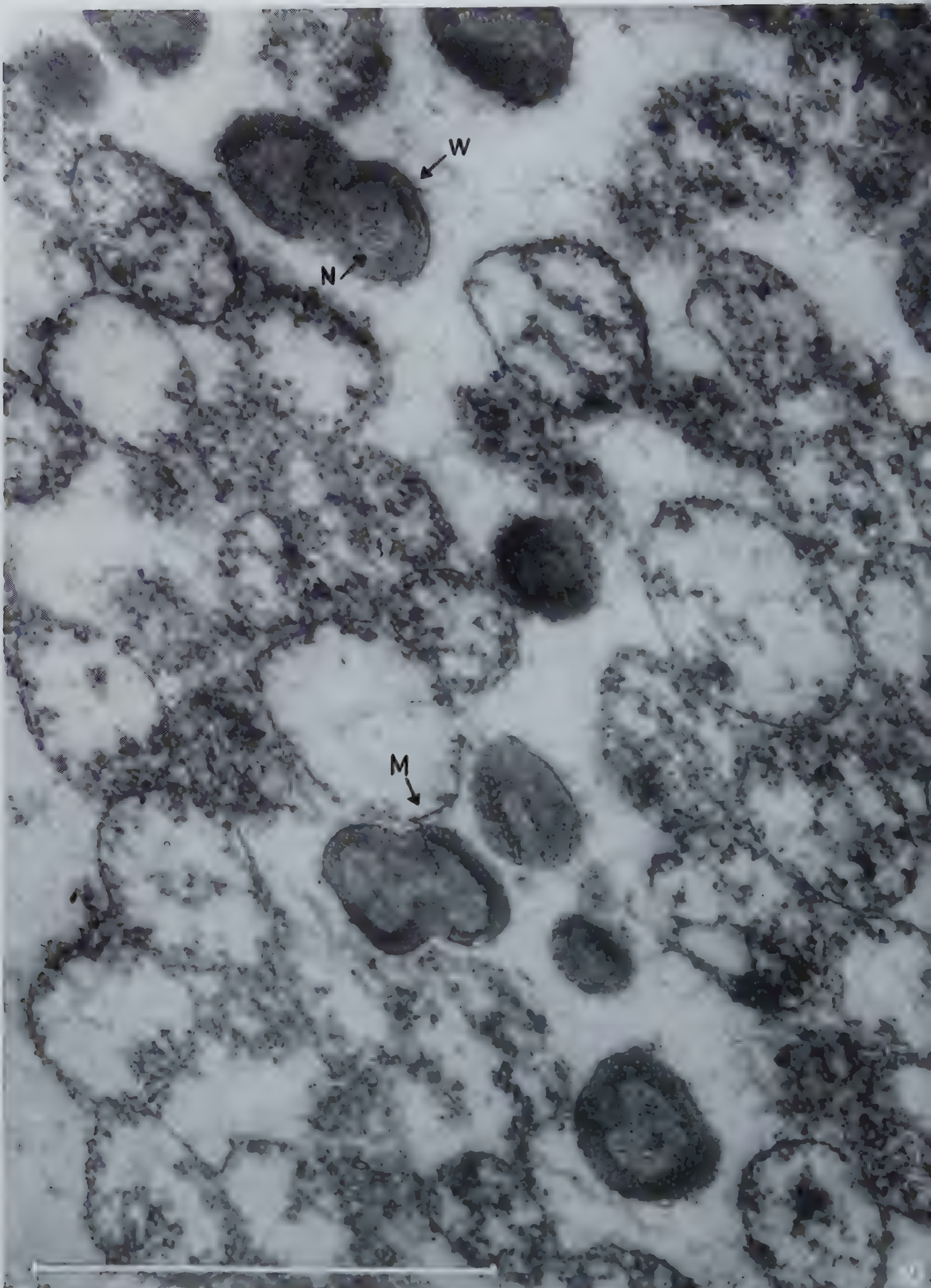
Fig. 6. *M. fermentans* after 24 hrs. on the collodion surface. At arrows cocci seem in continuity with other dense elements of the culture. Inoculation with a suspension. × 15,000.

Fig. 7. *M. fermentans* after 24 hrs. on the collodion surface. The cocci cannot well be distinguished from other dense elements. Inoculation with a suspension. × 15,000.

Fig. 8. Same as 6. At arrows strangely shaped cocci drawn-out towards other compact elements in the culture. × 15,000.

Fig. 9. Same as 10, but from central subsurface region of the colony. Note the structure in the vesicles of which the size can be compared to that of the cocci. There is a coccus at the arrow. × 39,000.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into minute parts, but this would not offer an explanation for the structures at b, c, d of the same order of magnitude as those supposed division parts. At the arrows A the outer surface of empty membranes can be noticed.

Vesicles can usually be distinguished from cocci by their surprisingly low content of solid structures (cf. fig. 10). However, we have found cocci in continuity with agglomerations of dense material, so that the question is raised whether such agglomerations are intermediate forms between vesicles and cocci. In the figs. 6 and 7 of *M.fermentans* (V26) areas are depicted which seem an intimate mixture of cocci and other dense parts. In fig. 8 unusually shaped cocci look as if drawn out towards those dense elements.

Frequently it is difficult to decide in a shadowed preparation between a coccus or another dense element. But in most ultra-thin sections it can be recognised how strongly the cocci differ from the average P.P.L.O. element, see fig.10. Fixed according to RYTER and KELLENBERGER (1958) the cocci appear characterised by a compact cell wall (arrow W fig.10), 150 Å across, dense cytoplasm and a nuclear element (arrow N fig. 10) of a striking structure, comparable to that encountered in other species of bacteria (VAN ITERSON, unpublished). The vesicles on the other hand are bounded only by a limiting membrane of 75 Å across (arrow M fig. 10), composed of two dense layers with a lighter zone in between (for more particulars see VAN ITERSON and RUYS, 1960). The amount of structure within the vesicles is variable and generally this content is very different from the cocci's differentiation in a cytoplasmic and nuclear region. However, in our micrographs of the vesicles with the largest amount of dense elements the distinction between the structure of the cocci and these vesicles is less clear. Sectioned vesicles of *M. fermentans* (V26) with a great deal of inner structure can be studied in fig. 9. Such areas were found in the subsurface central parts of the colonies. Vesicles transparent to the electron beam are in the sections considerably larger than the opaquer ones and often they are irregularly shaped (VAN ITERSON and RUYS, 1960). The more electron opaque structure the vesicle

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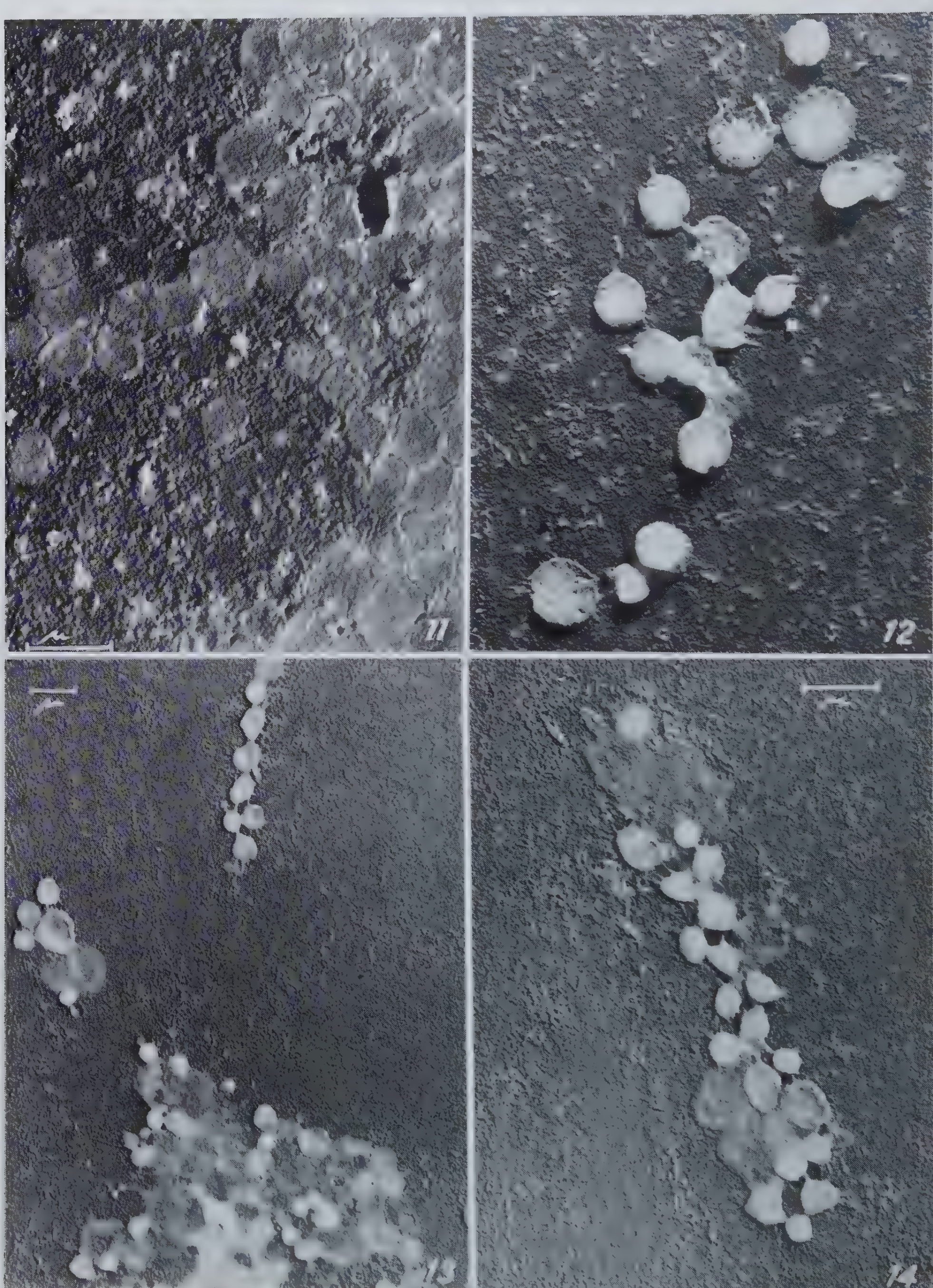
Fig. 10. *M. fermentans.* Ultra-thin section through a 4 days' old culture in agar. The vesicles are surrounded by a limiting membrane (M) of 75 Å and are comparatively poor in solid content. The cocci have a cell wall (W) of ca. 150 Å, dense cytoplasm, and a nuclear region (N) of striking structure. × 63,000.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contains, the more its size approaches that of a coccus. At the arrow in the lower part of fig. 9 a coccus can be recognised.

Fig. 11 is an example of the development of *M. salivarium* strain K 18. This colony, found at the agar side of the collodion membrane in a 5 days' culture, is clearly composed of flattened vesicles. But in fig. 12 in a colony fragment, which in a 2 days old culture adhered to the agar side of the membrane, some of the elements strongly resemble cocci. With the methods described in Part I (Ruys, 1960) this *M. salivarium* culture was recorded as being free of cocci. However, several months later, in the subcultures the flattened colonies typical of the cocci clearly appeared. At the time the figs. 11 and 12 were taken; the figs. 13 and 14 were made after a slightly different cultivation procedure, but there are no data available that this alteration influenced the result in the latter preparations. The inoculum on the membrane was covered with a block of plain distilled water agar, and after 7 days anaerobic cultivation the culture was kept aerobically at room temperature for 10 days. The striking feature in the figs. 13 and 14 are the compact bodies situated on empty membranes. They are flatter than the usual cocci, but are suggestive of a transitional stage towards the bacterial phase.

IV. **Discussion.**

In the preceding communication (Ruys, 1960) the improbability was discussed that the Gram positive coccoid elements in the P.P. L.O. cultures are contaminants in the usual sense.

Electron-microscopical study of various strains from the three

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Fig. 11. Typical colony of *M. salivarium*, 9 days' old, from the agar side of the collodion membrane. Individual units are distinct. Inoculation with a suspension. × 12,500.

Fig. 12. *M. salivarium* found at the agar side of the collodion membrane. The elements strongly resemble cocci though the culture was not supposed to contain these. Inoculation with a suspension. × 12,500.

Fig. 13. This culture of *M. salivarium* has been grown for 7 days on the collodion surface with a block of plain distilled water agar over it. The culture plate was then removed from the anaerobic jar and kept for 10 days at the normal atmosphere before the specimen was made. Note compact elements flatter than cocci. In this line of strain K 18 cocci were otherwise not yet observed. Inoculation with a suspension. × 6,250.

Fig. 14. The same as in fig. 13. × 10,500.

human *Mycoplasma* species revealed that in the colonies coccoid and vesicular elements can be intermingled in surprising intimacy (figs. 1, 2, 3, 5, 9, 10). In some of our shadowed specimens the cocci were seen in such close association with dense elements (figs. 6, 7, 8) that in these a clear distinction between various forms could hardly be made. In ultra thin sections of specimens in agar medium (figs. 9, 10) the cocci are found at various levels in the colonies (VAN ITERSON and RUYS, 1960). It is in such sections that the true bacterial anatomy of the cocci was discovered. In connection with the possibility that the vesicles are the L form of the cocci, it is of particular significance that the size of the coccoid bacteria approaches that of the vesicles with most internal structure (fig. 9); emptier vesicles are larger.

The figs. 12, 13 and 14, in our opinion, strongly suggest that compacter elements can develop connected to vesicles. The occurrence of such small vesicles with much dense structure (fig. 9) raises the question whether they represent transitional stages between the vesicular and bacterial forms.

The exact size of the cocci could not well be determined. In the shadowed electron micrographs they measure $\sim$0.45 μ across, but here these bodies are flattened on the membrane. In the sections the cocci are shrunk and then measure only $\sim$ 0.27 μ across. The size of the cocci may therefore be $\sim$0.3-0.4 μ which is in the range of magnitude of the large viruses.

Several authors, among whom may be mentioned MARTZINOSKY (1911), BEVERIDGE (1943), FREUNDT (1954), have already referred to coccoidal forms of P.P.L.O., but from most descriptions in the literature it is not clear whether the same elements are meant as the definitely bacterial forms here illustrated. But NELSON's studies (1936) on "coccobacilliform bodies" connected to the agent of fowl coryza could deal with elements comparable to the present cocci. NELSON (1939) feels that their extracellular manner of growth warrants their classification with the bacteria. CHU (1958a, b) emphasizes that the coccobacilliform bodies could be a form of P.P.L.O.

Reversion of P.P.L.O. to corynebacteria has been described e.g. by SMITH *et al.* (1957) and by WITTLER *et al.* (1956). Our coccoid elements, however, are not rod- nor club-shaped, they are much smaller and do not resemble diphtheroids. Besides, the strains isolated by WITTLER were katalase positive and fermented various

carbohydrates. Our coccoid elements were negative in both respects (RUYS, 1960).

In the literature it is suggested that in the propagation of P.P.L.O. an important part is played by so-called "minimal reproductive units", "elementary bodies", "elementary corpuscles", "coccoid elements", "spores" or "conidioids" etc. Frequently these elements are connected to a more complicated life cycle involving so-called "large bodies". As stressed in our study on the morphological details of the P.P.L.O. we have not encountered other elements than the vesicles and the cocci. Therefore, it is not possible for us to bring such a phenomenon as "the reproductive units" contained within a larger unit (cf. in particular KLIENEBERGER and SMILES, 1942) into connection with the present findings. This remains a question open to future research. From colonies in which the vesicles are the main or the only element it follows logically that vesicles are reproductive units (cf. VAN ITERSON and RUYS, 1960).

It appears of importance to compare the structure of *Mycoplasma* vesicles with those of a known L-phase variant of a bacterium. Sofar the electron micrographs of the mixed colonies of the three human *Mycoplasma* species suggest that these P.P.L.O. could be the L form of minute coccoid bacteria.

Summary.

Colonies of the three species of *Mycoplasma* of human origin were frequently found to contain coccoid elements. From electron micrographs it is deduced that the principle component of P.P.L.O. colonies are vesicular units of variable sizes. Usually these vesicular elements are largely vacuolar in nature. They then contrast strongly with the compact cocci. This study gives a number of data which seem to suggest that the coccoid elements and the P.P.L.O. forms belong together. Since the cocci are of definite bacterial structure the *Mycoplasma* species of human origin might represent the L phase variant of minute coccoid bacteria.

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THE INFLUENCE OF THE TOXINS SEPARATED BY ADSORPTION ON WOOD CHARCOAL FROM THE CULTURE OF *BACILLUS POLYMYXA* ON THE FERMENTATION OF SUCROSE

by

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(Received October 5, 1959).

It is a matter of common observation that in the bacterial cultures capable of producing 2,3 butanediol as one of the metabolites, the production of the diol takes place under specific conditions only and decreases in the course of time (SERENI, 1956). The quantity of 2,3 butanediol decreases and that of the acetoin increases. Apparently certain toxins are formed in the culture which inhibit further formation of 2,3 butanediol. It has been observed that if powdered charcoal is one of the initial constituents of the medium, the quantity of 2,3 butanediol is considerably increased (BAHADUR and RANGANAYAKI (1959a)). Hence it is quite probable that the toxins formed in the culture media are adsorbed by the charcoal powder, thus not interfering with the activity of the enzyme systems responsible for the formation of 2,3 butanediol in the culture.

The toxins adsorbed on charcoal powder have been separated by extracting the latter with acetone, absolute alcohol or petroleum ether in succession and the influence of these toxins on the formation of 2,3 butanediol and acetoin in the culture of *Bacillus polymyxa* has been studied (LEDINGHAM *et al.*, 1949; PORTER *et al.*, 1933; and BAHADUR and RANGANAYAKI, 1959b).

EXPERIMENTAL.

The culture medium contained: sucrose 10%, $MgSO_4$ 0.08%, K_2HPO_4 0.35%, KH_2PO_4 0.15%, $(NH_4)_2SO_4$ 0.17% and freshly prepared charcoal 2%. Fresh charcoal from burned wood was

washed repeatedly by boiling with water. After decanting the charcoal pieces were dried and powdered.

900 ml of this medium were introduced into a pyrex flask plugged with surgical cotton. 100 ml of an actively growing culture of *B.polymyxa* [C-3(2), National Research Council, Ottawa, Canada] were introduced. The flask was kept at room temperature (10-15° C.) during a fermentation period of 48 days. The culture was filtered through a quantitative filter paper and the charcoal powder was separated and dried over anhydrous $CaCl_2$ in a desiccator. The charcoal powder was then extracted with acetone by means of a Soxhlett apparatus. The extraction was carried out for 12 hours on a water-bath. The acetone was then evaporated on a waterbath and the residue dried in a vacuum desiccator. The same process was carried out with absolute alcohol and with petroleum ether in succession.

Four flasks (250 ml) were provided with 150 ml culture medium, containing sucrose 20 g, $MgSO_4$ 0.16 g, $(NH_4)_2SO_4$ 0.34 g and the required quantities of KH_2PO_4 and N NaOH solution to give a final pH of 6.4. After sterilisation as described each of these flasks was provided with 50 ml of an actively growing culture of *B.polymyxa*, which had been analysed as to its contents.

The estimations of acid and sugars - reducing and non-reducing - were carried out as described earlier (Bahadur and Ranganayaki, 1959a). The acetoin content was determined by the method of van Niel (1927) and the method of Moureu and Dode (1934) was employed for the estimation of 2,3 butanediol.

These cultures were incubated at 8-10° C. during 8 days. Then one was left as such whilst to the others one of the three residues was added. After a further fermentation during 10 days the cultures were analysed as to their contents. In Table 1 the results are reported, the data bearing on 200 ml of medium.

Discussion.

Some of the toxins formed in the culture of *B.polymyxa* and adsorbed by charcoal can be extracted with acetone. The residue obtained decreases the formation of reducing sugar and increases the sugar consumption of the organism. This causes the formation of alkaline material and inhibits the formation of acetoin. Though the quantity of 2,3 butanediol is the same as that in the control culture, the

TABLE 1.

Sugar, acid, acetoin and 2,3 butanediol in g.

Extractant	g equiv. of acid	Reducing sugar	Total sugar	Total sugar consumed	Acetoin	2,3 butanediol	
						g	% of sugar consumed
Control	0.0010	6.68	12.54	8.94	0.2880	0.363	4.06
Acetone	alkaline	3.40	9.30	12.18	0.0000	0.363	2.98
Abs. alcohol	0.0013	3.86	12.05	9.43	0.0000	0.198	2.09
Petroleum ether	0.001	7.07	12.49	8.99	0.0000	0.187	1.98

percentage yield of 2,3 butanediol calculated on the basis of sugar consumed during fermentation is decreased.

The toxin extracted by absolute alcohol causes a similar decrease in the formation of reducing sugar, but here the sugar consumption is not increased. However it causes a considerable decrease in the production of 2,3 butanediol.

The fraction extracted with petroleum ether effects a marked increase in the formation of reducing sugars. The yield of 2,3 butanediol is decreased.

All these fractions inhibit the formation of acetoin in the culture.

Summary.

Fractions from toxins adsorbed at charcoal have been separated by extraction. The acetone fraction causes decrease in the formation of reducing sugars and increase in sugar consumption, decrease in yield of 2,3 butanediol. The absolute alcohol fraction causes decrease in the formation of reducing sugar without affecting the sugar consumption and considerable decrease in the yield of 2,3 butanediol. The petroleum ether fraction increases the formation of reducing sugar without increasing the sugar consumption. It greatly decreases the production of 2,3 butanediol. All fractions inhibit formation of acetoin.

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LYSOGENICITY OF ATYPICAL STRAINS OF *SALMONELLA PARATYPHI* B

WITH OBSERVATIONS ON LATE D-TARTRATE FERMENTATION

by

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Introduction.

Shortly after the *Salmonella* types could be antigenically distinguished from each other by means of the receptor analysis, it appeared that the group of the strains of antigen formula 4, 5, 12; b–1,2 was not homogeneous. Strains were described by de Moor (1934, 1935) in Java which caused gastroenteritis and fermented d-tartrate. These strains were monophasic. A single d-tartrate positive strain causing gastroenteritis, but being diphasic, was found among 1017 strains by Kristensen and Boylen (1929). Similar strains were found in 1937 in an outbreak of gastroenteritis (Kristensen, 1938) in Denmark. In England many cases of gastroenteritis caused by d-tartrate positive strains, being monophasic, have been reported recently (Monthly Bull. Min. Hlth., London **14**, 73, 79, 83, 1955).

We will not go into the question here as to whether the monophasic d-tartrate positive strains are of human or animal origin (de Moor, 1956). All the d-tartrate positive strains caused acute gastroenteritis. They were all classified as one species, *S. java* by Kauffmann (1954, 1955). Brandis (1948) drew attention to the fact that the colony form of these strains corresponded with the particular colony form resembling a spoked wheel (Radspeichenform) which already had been observed by Graetz (1926) in the case of *S. typhimurium* and further *Salmonella* types causing gastroenteritis.

Phage typing entailed a new subdivision of *Salmonella* types. *S. typhosa* is subdivided by means of phage preparations which are adaptations derived from the same phage. A remarkable regularity was found when the phage types were examined as to their lysogenic properties. It was shown by Craigie (1946), Felix and Anderson (1951), and Anderson and Felix (1953) that in strains of the same Vi type the same natural phage is found. The possession of a natural phage is a very constant property of the bacterium; it is almost impossible to free the bacterium from its natural phage. *S. paratyphi* B appeared to contain a few natural phages only, i.e. phages I, IV, VI and II, which could be demonstrated alone or combined in the strains. The study of natural phages, however, can be useful for the demonstration of the homogeneity and characterisation of new types, and in certain cases as an auxiliary method in routine work (Scholtens, 1950, 1955, 1956, 1959). Boyd (1950) was able to set up a typing scheme, by which the identification of the natural phage of *S. typhimurium* is used as a routine method for the determination of phage types.

Here some strains of *S. paratyphi* B are described which harbour natural phages different from those usually found. This finding suggests that these strains should not be classified in the natural system; it may be more than a coincidence that these strains caused gastroenteritis.

Technique.

1. For the typing of the strains the technique described by Craigie and Felix (1947) was used.
2. The technique of the demonstration of the natural phages has been described elsewhere (Scholtens, 1959).
3. Heat sensitivity of the phages. The phage preparations were heated in a waterbath, at 56° C. for an hour. The preparations were titrated before and after heating. Each of a series of tenfold dilutions of the preparation was spotted on an agarplate inoculated with a sensitive strain.
4. Fermentation of d-tartrate. The prescription of Kauffmann (1951) was used.

Description of atypical strains.

The atypical strains to be described here originated from a family, where the children had slight diarrhoea and the mother was suf-

fering from a feverish illness affecting the hip joint. These 5 strains, called Buddenberg, formed a slime wall which was not very typical. According to FELIX and CALLOW they would have been typed Dundee. In the natural system of phage typing (SCHOLTENS, 1956, 1959) the strains could be differentiated from type Dundee by their reaction with the preparations of lytic spectrum e with which other strains of type Dundee do not react. However, they could not be classified in this system. Examination of their lysogenic properties demonstrated at once that these strains deviated from *S. paratyphi* B strains usually found. The filtrate of a pure culture of these strains produced plaques on plates inoculated with strain 3aB62 of *S. paratyphi* B. Some of the plaques had the appearance of phage I of *S. paratyphi* B and some had the appearance of phage IV of *S. paratyphi* B. From these plaques phages could be isolated which, as far as lytic spectrum and serology and plaque form are concerned, corresponded with phages I or IV. (No significance was attached to the fact that phage I of the Buddenberg strains did not wholly react to titre with serum anti phage I *S. paratyphi* B). Most of the plaques, however, were larger plaques, that differed from the plaques produced by the natural phages found in typical strains of *S. paratyphi* B.

On subculturing the larger plaques, phages were isolated which, as far as lytic spectrum is concerned, corresponded with phage VI. They reacted clearly with serums anti phage VI, and will be called phage VI, Buddenberg (Table 1).

TABLE 1.

Neutralisation of phage VI and phage VI Buddenberg with sera anti phage VI.

Phage	Serum	dilutions of serum 1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	Contr.
VI	27 anti phage VI	—	—	—	—	—	—	—	+3	+3
VI Buddenberg	27 anti phage VI	—	—	—	—	—	+1	+3	+3	+3
VI	452 anti phage hVI, F580	—	—	—	—	—	+1	+2	+3	+3
VI Buddenberg	452 anti phage hVI, F580	—	—	—	—	—	—	—	+1	+3

The reactions with the following two sera are reproduced:
1. Serum 27, prepared with phage VI from type Dundee.
2. Serum 452, prepared with phage hVI, F580, obtained from a mixed culture of a strain of type B.A.O.R. with a strain of type Beccles-Meppel (Filtrate 580 was used).

The phage VI from the true *S. paratyphi* B strains reacted better with the first serum than the phage VI Buddenberg. With the second serum the reverse was found.

Phage VI Buddenberg is thermolabile compared with phage VI *S. paratyphi* B. A rapid decrease in the number of plaques is al-

TABLE 2.

Sensitivity of heating at 56°C. of phages VI and VI Buddenberg.

Phage preparation	dilutions of phage preparations undil.	1 : 10	1 : 100	1 : 1000
VI, heated	cl	cl	cl-	+2
VI, unheated	cl	cl	cl-	+3
VI Buddenberg, heated	+1	—	—	—
VI Buddenberg, unheated	cl	cl	cl-	+2

The titre of preparations of phage VI (obtained from type Dundee) and phage VI Buddenberg was ascertained before and after heating during an hour at 56° C.

ready noticeable at 56° C. A comparative experiment can be seen in table 2. Rapid inactivation at 56°C. was never observed with phage VI *S. paratyphi* B.

The Buddenberg strains differ therefore from other strains of *S. paratyphi* B in lysogenic properties. In addition to phages I and IV they contain an atypical phage, phage VI Buddenberg. Moreover strains with natural phages I, IV and VI in combination have not yet been observed in the case of *S. paratyphi* B.

As far as fermentation of d-tartrate is concerned the Buddenberg strains differed from *S. java*. They were sent to Dr. KAUFFMANN who found that they were d-tartrate negative after 24 hours, but that two of the strains in some of ten test tubes fermented d-tartrate after a fortnight. From these tubes it was possible by subculture from single colonies to obtain variants of the strains which promptly fermented d-tartrate. On repeating these tests in Utrecht d-tartrate positive variants could be cultivated from all strains after four weeks incubation.

In valuating these results it must, however, be pointed out that only two of the five strains fermented d-tartrate after a fortnight in several test tubes and that some true *S. paratyphi* B strains will also ferment d-tartrate after a longer period of incubation, as shown in the following experiments. Ten test tubes containing d-tartrate were inoculated with all the paratyphoid B strains received for typing and examined after three weeks. Strains were found which fermented d-tartrate in some of these ten test tubes. From these, variants of *S. paratyphi* B which fermented d-tartrate could be cultivated, although only after several passages. The strains from

which these variants were obtained formed a typical slime wall and were d-tartrate negative after 2 × 24 hours' incubation. They belong to types Taunton-Kampen, 3aI-Leeuwarden and Sittard. Strains of the first two types came from cases of enteric fever occurring in an environment where several cases of febris paratyphoidea had been observed caused by the same type. The strain of type Sittard, however, appeared to be isolated from one of several cases of gastroenteritis caused by the same type.

Although the Buddenberg strains, as far as pathogenesis is concerned, resemble *S. java* they cannot be distinguished from *S. paratyphi* B on fermentation of d-tartrate.

A second set of remarkable *Salmonella* strains was found in two healthy children during an epidemic of Sonne dysentery. The strains did not react with phages $0_{1,2,3}$ of FELIX and CALLOW, and furthermore reacted with typing phages d and e only. On examination they appeared to contain natural phages corresponding with phage IV as far as plaqueform and lytic spectrum were concerned. On serological examination, however, these phages were neither neutralised by anti IV serum of high titre, nor by sera VI, II and I. These strains showed a typical slime wall and did not ferment d-tartrate, not even when ten test tubes were inoculated and observed for a month.

These and other strains which fall outside the natural system of phage typing always appear to be isolated from healthy persons or in cases of gastroenteritis or in atypical cases of illness. Atypical strains came from atypical cases.

The opinion was put forward in a previous article (SCHOLTENS, 1956) that the series of phage types in the natural system are subspecies of *S. paratyphi* B. It is probable that the whole group of bacteria with antigen structure 4, 5, 12; b–1,2 consists of many more or less related sub-species in which it is perhaps possible that the dividing line between the producers of febris paratyphoidea and gastroenteritis does not wholly coincide with the dividing line between d-tartrate positive and d-tartrate negative strains. The strains described above with atypical natural phages belong presumably to a sub-species standing close to the true *S. paratyphi* B strains.

Just as with strains of the antigen formula 1, 4, 5, 12 i 1,2 (BOYD, 1950) so with strains of antigen formula 1, 4, 5, 12 b 1,2 some „phage types" can be discerned by means of the identity of the natural

phage. However, in strains with the antigen structure 4, 5, 12; b–1,2 attention is centred upon the human strains that cause paratyphoid fever. These strains show only a slight variation in the identity of their directly demonstrable phages. Strains similar to those described above, are rarely isolated, and strains of *S. java*, which show a very great variability in the identity of their natural phages, are diagnosed with the fermentation of d-tartrate.

With strains with antigen formula 1, 4, 5, 12, i 1,2 the case is different (Boyd, 1950). Here strains of various origins are considered and no single group predominated. In this connection a greater variation in the identity of natural phages is found.

Summary.

Atypical strains of *S. paratyphi* B have been described which caused gastroenteritis or were found by chance in healthy persons. These strains possessed atypical natural phages.

The late fermentation of d-tartrate by some strains of *S. paratyphi* B was examined.

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STUDIES ON THE CORRELATION BETWEEN TOXIGENICITY AND LYSOGENICITY OF *CORYNEBACTERIUM DIPHTHERIAE*

by

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In 1951 FREEMAN was the first to describe a correlation between the capacity of a diphtheria bacterium to form toxin, and the lysogenic property. However, only a small number of bacteriophages proved capable of converting non-toxigenic diphteria bacteria into toxigenic ones by lysogenization. Non-toxigenic, lysogenic diphtheria bacteria proved to be of common occurrence; the lysogenicity of many toxigenic strains could not be demonstrated.

Several investigators have attempted to answer the question to what extent a direct relation between toxigenicity and lysogenicity is involved in the phenomenon mentioned, for mutation followed by selection of toxin producing mutants in a culture of non-toxigenic diphtheria bacteria might account for the above phenomenon as well.

FREEMAN and MORSE (1952) were able to demonstrate the phenomenon, starting from "single-cell" cultures; no preformed toxin proved to be present in the non-toxigenic bacteria. PARSONS and FROBISHER (1951) argued against the selection of mutants the fact that the toxigenic clones are far more frequent than is to be expected according to a normal chance of mutation; moreover, in non-toxigenic diphtheria cultures without phages toxigenic colonies were never met with. Likewise the results of investigations by GROMAN (1953, 1953a, 1955), BARKSDALE and PAPPENHEIMER (1954), and HEWITT (1954) all plead against the selection of a mutant. Most convincing appears to be the finding that within 20 minutes after addition of phage filtrate to the bacterial culture

sometimes up to 7 % of the bacteria had been converted into toxigenic ones (Groman, 1953a).

Transduction, *i.e.* conveying of the genetic properties of one bacterium onto the other by means of a phage, was ruled out as an explanation of the phenomenon of conversion by Groman (1955).

So far nothing is known of the mechanism of conversion. Possibly the lodging of the prophage on a specific "locus" of the hostcell results in the phaenotypic property of toxin formation (Groman, 1955). Viewed in the light of the definition of a prophage given by Lwoff (1957), this seems quite plausible. On the other hand, it is also possible that this hereditary property is only present in the prophage, independent of the type of hostcell. However, this is not in accordance with the findings of Groman (1956) and Parsons (1955), which show that the manifestation of the converting property of a phage is dependent on the hostcell.

Hewitt (1952) has pointed out the possible epidemiological significance of the phenomenon of conversion. According to Hewitt the frequent occurrence of diphtheria phages in nature, combined with the converting property of a number of these, can account for the variation in virulence of infecting organisms as well as furnish evidence of the significance of non-toxigenic diphtheria bacteria for epidemiology. In the first place we have tried to answer the question put by Hewitt in this connection, *viz.*, whether diphtheria strains, isolated from patients, are lysogenic by phages to which they, originally non-toxigenic and non-lysogenic, have been exposed. For this purpose the first throat swabs of a number of diphtheria patients, who could be regarded as isolated cases, were examined for the presence of non-toxigenic diphtheria bacteria. At the same time the isolated toxigenic strains were examined for lysogenicity.

In order to gain an impression concerning the phage-specificity of the converting property, cross-neutralization and cross-immunity tests were performed with a number of phages, one of which (β) was capable of conversion. Groman and Eaton (1955) could not demonstrate any serological differences between phage β and phage γ, which latter is incapable of conversion. From this they drew the conclusion that the converting property is phage-specific, although cross-immunity tests did reveal differences between the two phages.

It was not possible to demonstrate the (possibly defective) lysogenicity of diphtheria strains by means of U.V. irradiation, as had proved feasible for coli strains (Jacob, Fuerst and Wollman,

1957). Along this line, therefore, no data could be obtained on the presence or absence of the lysogenic property in a strain of *C. diphtheriae* (Mouton, 1959).

There is certainly no question of a constant relation between toxigenicity and lysogenicity, in view of the occurrence of non-toxigenic, lysogenic diphtheria strains. However, the possibility remains that the toxigenicity of a diphtheria bacterium does depend on the lysogenic property. For the fact that many toxigenic diphtheria strains cannot be demonstrated to be lysogenic may be due to lack of a strain susceptible to the phage concerned. We have approached this problem by attempting to free toxigenic diphtheria strains from their phages and proceeding to ascertain whether the capacity to produce toxin had been lost together with the lysogenic property.

Material and Methods.

a. Bacterial cultures. The strains of *C. diphtheria* used in our investigation were for the greater part isolated in the Regional Public Health laboratories at Heerlen and Rotterdam. For details on these strains, nearly all belonging to the gravistype, reference may be made to a previous publication (Mouton, 1959). The strains C4 and C4 (β), with which the phenomenon of conversion was demonstrated, were obtained from the Department of Microbiology, School of Medicine, University of Washington, Seattle, U.S.A. The strains were kept on Loeffler serum and transferred every 4–6 weeks.

b. Phages. Out of 42 strains, including C4 (β), 6 phages were isolated according to techniques in which use was made of the inducing effect of U.V. irradiation, and which were already described in detail elsewhere (Hewitt 1952; Thibaut and Fréodericq, 1952; Mouton, 1959). The presence of phages was demonstrated by means of Gratia's double agarlayer method (1936). The broth medium used was Heart-Infusion-Broth (Difco) (H.I.B.); the agar media consisted of H.I.B. + 1,5 % and 0,7 % agar respectively. For the preparation of bacteriafree phage suspensions Jenaer Ganzglas Bakterienfilter 3 G 5 m were adopted. Loss by filtration was not demonstrable if beforehand the filter had been treated with 10 ml H.I.B.

Phage suspensions with high titres were obtained according to

the method of SWANSTROM and ADAMS (1951). By this method titres of 10^8–10^9 could be reached. Pulverization of the scraped-off soft agarlayer in a mortar, prior to extracting it with H.I.B. proved to be of great influence on the height of the titre.

Comparative experiments have demonstrated (MOUTON, 1959) that when a deep-freezer of —70°C. (cf. GROMAN and BOBB, 1955) is not available, preservation of phage filtrates at 4°C. is preferable to freezing at —35°C. However, even then fresh phage filtrates with high titres should be prepared at regular intervals.

c. Phage-resistant, lysogenic strains. These strains were prepared in H.I.B. The phage suspensions used for the purpose had a but moderately high titre ($\pm 10^3$); this was caused by the small number of transfers a temperate phage can undergo on a susceptible strain without acquiring greater virulence with respect to this strain (cf. GROMAN and EATON, 1955). The lysogenicity of the phage-resistant cultures thus obtained was demonstrated with the aid of the inducing effect of U.V. irradiation (MOUTON, 1959). It could not be ascertained whether non-lysogenic, phage-resistant bacteria were still present in these cultures. On account of the growth in clumps of diphtheria bacteria, isolation of a single colony will give no certainty either that one is dealing with a purely lysogenic strain.

d. Preparation and application of phage antisera. Two rabbits were each subjected to 8–10 subcutaneous injections with the filtrate of the phage against which antiserum was to be prepared. In the case of a converting phage 50–100 E antitoxin/ml was added to the filtrate to be injected in order to neutralize any toxin present. The neutralization-indices (K) of the sera with respect to the phages were determined according to the formula of HERSHEY, KALMANSON and BRONFENBRENNER (1943):

$$K = 2{,}3\ D/t\ .\ \log p_0/p,$$

where p_0 = quantity of phage prior to start of reaction
p = quantity of phage at t minutes after start of reaction
D = final dilution of the serum in the phage-serum mixture.

The p- and p_0-values were determined according to ADAMS (1950). With the aid of the K-values it can be established whether given phages are serologically related. Relationship between two phages may be assumed if the K-values of serum prepared against one phage are the same or nearly the same for both phages (ADAMS, 1950).

e. Toxigenicity tests. Use was made of a modification of ELEK's method (1948). The modification consisted in the fact that the filter paper with antitoxin (1000 E/ml) was placed on the proteose peptone serum agar medium which was prepared beforehand and kept at 4°C. The cultures to be examined were inoculated in strips perpendicular to it up to 0.5 cm distance from the paper. The proteose peptone serum agar medium was the one adopted by FROBISHER, KING and PARSONS (1951) for this purpose.

If the outcome of this toxigenicity test was negative or doubtful a guinea-pig test was done according to RÖMER (1909), with this difference that the antitoxin was simultaneously administered intraperitoneally to the control guinea-pig and not 24 hours previously (cf. FREEMAN, 1951).

In order to isolate in a simple manner a non-toxigenic clone from a culture which might consist of a mixture of toxigenic and non-toxigenic bacteria use was made of the method already applied before by GROMAN (1953a). From a culture to be examined an inoculation was made onto a medium (P.P.S.A.) consisting of proteose peptone agar, 10 % horseserum and 50 E antitoxin, and prepared a few hours in advance. After 2 days' incubation followed by a few days at + 4°C. haloes could be observed round the colonies, which consisted of toxin producing bacteria. Colonies of non-toxigenic bacteria did not display these haloes.

EXPERIMENTS.

a. **Investigation into the occurrence of non-toxigenic diphtheria bacteria in patients with diphtheria.** This was an attempt to trace to what extent the phenomenon of conversion is of significance for the epidemiology of diphtheria. The frequent occurrence of non-toxigenic bacteria during an epidemic (cf. BARKSDALE and PAPPENHEIMER, 1954) might be explained by assuming conversion in vivo, at any rate if the increase in the number of carriers of non-toxigenic bacteria is primary.

In the course of an epidemic most cases of diphtheria are bound to be caused by contacts with patients or carriers of toxigenic bacteria. The possibility remains, however, that in isolated cases conversion in vivo is playing a part. For this reason the first throat swabs of a number of patients who could be regarded as isolated cases of diphtheria were examined for the presence of non-toxigenic

diphtheria bacteria side by side with the toxigenic ones. For this purpose a number of colonies grown on Clauberg medium were examined for toxigenicity. The colonies, 495 in all, derived from 26 throat swabs, all proved to consist of toxin producing diphtheria bacteria.

In consequence of a publication by Parsons and Frobisher (1951) on the occurrence of non-toxigenic diphtheria bacteria in convalescents, the examination for non-toxigenic bacteria on a few of the patients examined in the initial stage of the disease was repeated 3–4 weeks later, also without results.

For practical reasons the search for non-toxigenic diphtheria bacteria could not be extended to contacts of patients. A large-scale investigation of this sort may be more apt to furnish data which can give us an insight into some epidemiological aspects of the phenomenon of conversion.

b. **Investigation into the phage specificity of the converting property.** Apart from phage β, derived from strain C4 (β), phages were isolated from 5 gravis strains. The nomenclature by Fahey (1952) was followed in the terms given: phage 11/1, was isolated from strain 11 and is capable of lysing strain 1. The isolated phages were the following: 11/1, 13/1, 16/1, 22/1 and 39/1. Preparation of the strains lysogenic by these phages was performed according to the method described above. None of the 5 phages proved capable of converting strain 1 (C4) to toxigenicity.

Having at our disposal 6 phages, one of which was capable of conversion, we have attempted to answer the question as to the phage specificity of the converting property by conducting cross-neutralization and cross-immunity experiments.

In the cross-neutralization tests use was made of the virulent mutants of the isolated phages, obtained by repeated transfers of the temperate phage on the susceptible strain in H.I.B. Only with the virulent mutants titres sufficiently high to immunize rabbits could be reached. It appears from the data furnished by Burnet and Lush (1936), Adams (1950) and Bertani (1958), that virulent mutants and mutants with a modified hostrange are serologically indistinguishable from temperate phages.

Table 1 contains the K-values which resulted from the cross-neutralization tests between the 6 available phages and antisera against 5 of these. In determining the K-values we have observed Adams' (1950) condition of maximum 99 % neutralization and

minimum 90 % neutralization so as not to invalidate the formula from which K is calculated. The values given in Table 1 are those resulting from neutralization tests with a maximal dilution and a minimal neutralization time, in which at least 90 % neutralization occurred.

TABLE 1.

K-values of phage antisera with respect to phages; results of experiments in which the highest possible serumdilution and the shortest time of neutralization were applied, to give rise to a neutralization percentage of at least 90 %.

serum / phage	anti B	anti 39/1	anti 16/1	anti 22/1	anti 11/1
B	8.18	0.48	—	—	—
	7.49				
	7.49	0.52	—	—	—
	6.62				
39/1	—	0.84	0.54	1.58	0.77
	—	1.32	0.47		0.86
16/1	—	0.50	1.13	1.55	0.74
	—	0.53	0.82	1.73	0.91
22/1	—	—	—	1.67	0.81
	—	—	—	1.67	
				1.60	0.97
				1.69	
11/1	—	—	—	—	0.77
	—	—	—	—	0.76
					0.85
13/1	—	—	—	—	—
	—	—	—	—	—

— : K < 0.38.

Although DELBRÜCK (1946) attached little value to cross-immunity tests for the purpose of classifying phages, he nevertheless came to the conclusion after comparison of the results of these tests on coliphages with those of cross-neutralization tests that there were correspondences. Although nothing is known about the nature of the immunity of a lysogenic bacterium against the activity of a particular phage (BERTANI, 1958), cross-immunity tests may yet give us indications of a possible correspondence between immunological and antigenic characteristics.

The results of the cross-immunity tests are given in Table 2.

TABLE 2.

Cross-immunity of lysogenic strains and their phages.

phage \ strain	C4Rβ	C4R39/1	C4R16/1	C4R22/1	C4R11/1	C4R13/1
β	—	—	+	+	+	+
39/1	+	—	—	—	—	—
16/1	+	—	—	—	—	—
22/1	+	—	—	—	—	—
11/1	+	—	—	—	—	—
13/1	+	—	—	—	—	—

+ : lysis
—— : no lysis
R : resistant to

Inspection of the results of the cross-neutralization and cross-immunity tests will reveal a certain correspondence. There appears to be an, if slight, affinity between phages β and 39/1, and a certain affinity between phages 39/1, 16/1, 22/1 and 11/1. Only phage 13/1 gives divergent results in the two series of experiments.

Groman and Eaton (1955) failed to demonstrate any antigenic differences between phage β and phage γ, which is incapable of conversion. So phages β and γ are certainly not identical, not to mention the converting property of phage β. It will be clear that study of a number of phages of which only one is capable of conversion, admits of no definite conclusion on the relation between antigenic properties, immunological characteristics and converting property. We should like to advance, however, also on account of experiments by Groman and Eaton, that it is possible to demonstrate that there are antigenic or immunological differences between a phage with converting property with respect to a specific strain and phages lacking this property.

c. **Experiments intended to isolate non-lysogenic from lysogenic, toxigenic diphtheria bacteria.** With the aid of a highly virulent mutant of phage β, Groman (1955) succeeded in isolating bacteria of strain C4 from a culture of C4 (β) bacteria. The mechanism of this reversion to a non-toxigenic and non-lysogenic bacterium is unknown. Groman's method has the disadvantage that it can only be applied if the lysogenicity of the toxigenic strain in question is a certainty

and if the phage concerned and a corresponding non-toxigenic strain on which a phage can be made more virulent by transfers, are available. Search was therefore made for a different method which would offer better promise of success.

In a lysogenic culture a cell may occasionally pass into the non-lysogenic state. By adsorption of the phages present in the culture, released by spontaneous lysis of lysogenic bacteria, these cells will be lysed or become once more lysogenic. An equilibrium will thus be attained between lysogenic and non-lysogenic, phage-susceptible bacteria, dependent on the extent to which reversion to susceptibility to the phage occurs and on the degree of reinfection (Bertani, 1958). The latter author gave a survey of the investigations made with a view to obtain non-lysogenic, phage-susceptible bacteria from a lysogenic culture.

Clarke (1952) and Lwoff (1953) were successful with *B. megatherium* by means of a method based on the fact that the presence of Ca-ions in the medium is essential for the phage to penetrate into the bacterium. The same holds good for *C. diphtheriae*. Clarke added Na-citrate to the medium. Lwoff used oxalate to eliminate the influence of Ca-ions and thus to prevent infection of the non-lysogenic bacteria in the culture. After 61 and 24 transfers resp. of the lysogenic strains in such a medium non-lysogenic cultures were obtained.

After ascertaining the inhibiting effect on the phage-activity of a medium consisting of H.I.B. + 3 ‰ Na-citrate, 15 toxigenic diphtheria strains, 4 of which were known to be lysogenic, were daily transferred in this medium. After a number of transfers ranging from 40 to 75 (C4 (β)), the cultures were inoculated onto H.I.B. agar media, after which toxigenicity of 20 colonies per strain was determined. These tests all turned out positive. In the cultures of strains known to be lysogenic, free phages could be demonstrated after inoculation onto H.I.B. This method failed to gain the desired result, therefore.

Groman and Bobb (1955) established that the non-ionic detergent Tween 80 in concentrations of 0.1 % or higher completely inhibits the adsorption of phage β to C4 bacteria. In some other bacterium-phage systems this effect could not be demonstrated. Nevertheless we were of the opinion that it would be worth while to add Tween 80, as a substance inhibiting phage-adsorption, to a medium in which toxigenic strains would have to be transferred in an attempt to obtain

non-lysogenic mutants. Since the activity of Tween 80 is based on a modification of the bacterial surface, which had proved reversible, the chance that repeated transfers of lysogenic bacteria would result in a non-lysogenic, but phage-insusceptible strain did not seem great.

The inhibition of the adsorption of phage β to C4-bacteria by Tween 80 could be demonstrated. Tween 80 proved to be neither phagocide nor to influence bacterial growth.

The transfer experiment in H.I.B. + 0.1 % Tween 80 (T.H.I.B.) was restricted to toxigenic strains known to be lysogenic. After three single-colony isolations of strains C4 (β), 11, 13, 16, 22 and 39, seventy nine successive transfers were performed in T.H.I.B. The cultures of the 79th transfer were inoculated onto P.P.S.A.-media. After two days at 37°C. and five days at 4°C. the colonies grown on the media were examined for the formation of haloes. After an additional two alternating transfers in T.H.I.B. and P.P.S.A. non-toxigenic bacteria, which in all respects (type, phage-susceptibility pattern) were identical with those of strain C4, were isolated from the original C4 (β) culture.

In the same way non-toxigenic bacteria which belonged to the mitis type and of which the lysogenicity could not be demonstrated, were isolated from the culture of the toxigenic gravis strain 13. However, these bacteria (strain 13A) were insusceptible to phage 13/1; adaptation proved impossible.

Passage of gravis strain 11 also produced non-toxigenic bacteria of the mitis type. This bacterial culture proved to contain still another phage, however. This could not be removed by alternating T.H.I.B.-P.P.S.A.-transfers. The culture resistant against this phage (D) proved to be non-toxigenic as well (11A). Phage D was not identical with phage 11/1, since phage D could not be adapted to C4. Phage 11/1 proved inadaptable to strain 11A. Although strain 11A was likely to be lysogenic on account of phage D, its lysogenicity could not be demonstrated.

Transfers of strains 16, 22 and 39 merely produced toxigenic, lysogenic bacterial cultures.

It is therefore indeed possible by this method to obtain the non-lysogenic, phage-susceptible non-toxigenic strain C4 from strain C4 (β). The fact that 79 T.H.I.B.transfers of strains 16, 22 and 39 did not give rise to the formation of a culture of non-lysogenic bacteria may find its cause in a specificity of the phage adsorption-

inhibitive effect of Tween 80, but also in a too small number of transfers of these strains.

The mutation of strains 11 and 13 to non-toxigenic mitis strains might also be due to a direct action of Tween 80 on the bacteria, unconnected with the inhibition of phage adsorption. But in that case the fact remains that on mutation these strains lost both their toxigenic and their lysogenic properties. The insusceptibility of strains 11A and 13A to phages 11/1 and 13/1 does not justify a definite conclusion on a direct relation between toxigenicity and lysogenicity in these strains. Yet we are of the opinion that above results indicate that the two properties are also correlated in strains 11 and 13. As long as a possible lysogenicity of any toxigenic strains remains obscure we may, however, draw no conclusions from this in favour of the hypothesis that in all toxigenic diphtheria strains toxigenicity is dependent on lysogenicity.

Discussion.

The experiments described under *a* yielded no results indicating an epidemiological significance of the phenomenon of conversion. However, the fact that non-toxigenic bacterial mutants in vitro could be isolated from toxigenic strains, if only by artificial operation, raises the question whether this may also happen in vivo. Direct indications of this are lacking, but data of Okell (1929), Chason (1936), Stebbins (1940), Gill (1940) and Frobisher *et al.* (1947) certainly are in favour of it. The frequent occurrence of non-toxigenic diphtheria bacteria during an epidemic (cf. Barksdale and Pappenheimer, 1954) might also be caused by a mutation of toxigenic into non-toxigenic bacteria. A mutation of this sort might consist in a reversion of lysogenic, toxigenic into non-lysogenic, non-toxigenic bacteria and a selection of the latter. There is no reason to assume that loss of the lysogenic property of a bacterium is not just as liable to occur in vivo as in vitro. A reversion of lysogenic, toxigenic into non-lysogenic, non-toxigenic bacteria in vivo followed by a selection of the latter by unknown factors (influences of environment?, phage-antibodies?) might account for the prevalence of non-toxigenic diphtheria bacteria in the course of an epidemic.

The results of recent experiments by Anderson and Cowles (1958) do indeed argue in favour of the significance of phage-anti-

bodies for reversion in vivo. By means of transfers of a toxigenic, lysogenic diphtheria strain in a medium with phage-antiserum these workers succeeded in obtaining in vitro a non-toxigenic strain that was susceptible to and converted by a phage isolated from the original lysogenic strain. This publication does not make clear whether the relationship between toxigenicity and lysogenicity of the strain examined was known.

However, this has not provided an answer, either, to the question whether the toxigenicity of diphtheria bacteria is always dependent on the lysogenic property. Extension of the experiments in vivo (on guinea-pigs) by ANDERSON and COWLES to toxigenic strains that can or cannot be demonstrated to be lysogenic, but in which no connection between the two properties can be established by means of other, non-toxigenic strains, is certainly desirable if this problem is to be solved.

When we consider the outcome of the cross-neutralization and cross-immunity tests together with the results of the experiments by which in a number of any toxigenic, lysogenic strains a relation between toxigenicity and lysogenicity was made plausible, we will note a certain discrepancy. For the former series of experiments reveal marked differences between a phage whose converting property had been demonstrated, and a few other phages. From the latter experiments it might be concluded that phages 11/1 and 13/1 also have influence on the capacity to form toxin.

GROMAN (1956) gave three possible explanations of the phenomenon that phages isolated from a non-toxigenic strain may possess converting properties with respect to a different non-toxigenic strain. The third explanation, to the effect that the converting action of a phage can be suppressed, appeals most to us. Our grounds for this were expounded in a previous publication (MOUTON, 1959).

More specified this explanation implies that a specificity is assumed for the capacity of a phage to cause conversion. Along with the phage-susceptibility a conversion-susceptibility of the bacterium to a phage would also play a part then.

The fact that antigenic and immunological differences were nevertheless found between phage β and the other phages need not be surprising, since phage β and the other phages differ in the converting property with respect to strain C4. The host-specificity of the converting property might be reflected in certain antigenic or

immunological properties of the phage. The results of our cross-neutralization and cross-immunity tests are in accordance with this. Both the findings of PARSONS (1955) and GROMAN (1956) and the results of our own work can be explained by this hypothesis.

Summary.

Description of a study of some aspects of the correlation between toxigenicity and lysogenicity in *C. diphtheriae.*

The question as to the epidemiological significance of the phenomenon of conversion had to remain unanswered.

Cross-neutralization and cross-immunity tests with 6 phages, antisera against 5 of these, and strains lysogenic by these phages, showed antigenic as well as immunological differences between a phage with converting property with respect to one specific strain and some phages lacking this property.

By means of transfers of lysogenic, toxigenic strains in a medium to which Tween 80 was added as a substance inhibiting phage-adsorption, non-toxigenic not demonstrably lysogenic strains could be isolated in three out of six cases.

A relationship between toxigenicity and lysogenicity, in two of the three strains previously unknown, is assumed. The possibility of the occurrence of this reversion in vivo is discussed.

Reversion and selection of non-lysogenic, non-toxigenic mutants in vivo is suggested as an explanation of the frequent occurrence of non-toxigenic diphtheria bacteria during an epidemic.

In conclusion the hypothesis is advanced that a conversion-susceptibility of non-toxigenic diphtheria bacteria might exist side by side with a phage-susceptibility. The host-specificity of the converting property might be reflected in certain antigenic or immunological properties of the phage.

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THREE NEW ARIZONA TYPES ISOLATED FROM NORMAL REPTILES
(5:29:30; 16:22:34; and 20:29:25)

by

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The organisms to be described were isolated from the feces of apparently normal snakes which were confined in a zoo. Culture 5241–58 was isolated from a *Constrictor constrictor*, culture 5243–58 from a *Eunectes murinus*, and culture 5245–58 from a *Crotalus viridis*. The biochemical characteristics of the organisms were typical of those of the Arizona group (LE MINOR, FIFE and EDWARDS, 1958) with the exception that culture 5241–58 produced indol and culture 5245–58 failed to liquefy gelatin. When isolated, the cultures were only feebly motile and many passages in semisolid medium were required before a satisfactory examination of the H antigens could be made. The serologic characteristics of the cultures were as follows:

5243–58. The culture was agglutinated to the titre of Arizona O group 5 serum but in absorption tests reduced the titre of the serum only by 50 per cent. Since it was agglutinated by no other Arizona O serum, it was assigned to O group 5 in spite of the non-identity of the O antigens with those of the standard O group 5 strain. The fact that the culture was somewhat rough may account for its failure to exhaust O5 serum of agglutinins. The H antigens were diphasic. Phase 1 was agglutinated to the titre of, and in absorption tests removed all agglutinins from Arizona H29 serum. Phase 2 was agglutinated to the titre of Arizona H30 serum. In absorption tests it reduced the titre of the serum from 20,000 to 200. The organism was assigned the antigenic formula 5:29:30. A second culture of this

type was recovered from a snake of the same species (*Eunectes murinus*) kept in the same cage.

5241–58. The culture was agglutinated to the titre of, and in absorption tests removed all agglutinins from Arizona O16 serum. The H antigens were diphasic and phase 1 was found to be identical with Arizona H22 by agglutination and absorption tests. Phase 2 was agglutinated to 25 per cent of the titre of Arizona H34 serum. In absorption tests it reduced the titre of the serum by 50 per cent. It was agglutinated by no other Arizona H serum. Although the antigen of phase 2 is not identical with the H antigen of the standard strain of H34, in the interest of simplicity, the culture was designated by the antigenic formula 16:22:34.

5245–58. The O antigens of the culture were identical with those of the standard strain of Arizona O group 20. The H antigens were diphasic and phase 1 was agglutinated to the titre of Arizona H29 serum and in absorption tests reduced the titre of the serum from 32,000 to 500. A satisfactorily motile culture of phase 2 which flocculated well in H sera was never obtained in spite of repeated trials. The most satisfactory culture which was obtained flocculated only to 10 per cent of the titre of Arizona H25 serum. In absorption tests this culture reduced the titre of H25 serum from 8000 to 2000. An H serum was prepared from phase 2 of 5245–58. While this flocculated the homologous strain only in a dilution of 3,200, the standard strain of Arizona H25 was agglutinated to a dilution of 12,800. Absorption of 5245–58 phase 2 serum by the standard strain reduced the titre from 3,200 to 400. The organism was assigned the antigenic formula 20:29:25.

Summary.

Three new Arizona serotypes (5:29:30; 16:22:34; and 20:29:25) are described. All were isolated from the feces of apparently normal snakes in a zoo. The 16:22:34 type produced indol and the 20:29:25 type failed to liquefy gelatin. Otherwise, the cultures possessed biochemical characteristics typical of the Arizona group. The details of the serologic reactions of the cultures are described.

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DIE GRAPHISCHE DARSTELLUNG DER VIRULENZ BEI ASCITES-TUMOREN (VIRULENZCURVEN-METHODE)

von

W. A. COLLIER und **M. DE WIT**

(Empfangen 25. August 1959).

Nicht nur bei den durch Virusarten verursachten Tumoren, wie ROUSsarkom, SHOPEpapillom usw., sondern auch in allen jenen Fällen, in denen man es mit einer ,,Tumorcelle" zu tun hat, ist man durchaus berechtigt, von Virulenz zu sprechen. In jedem Fall hat man einen ,,Krankheitserreger" vor sich, mag es sich nun um ein Virus handeln oder um die Tumorcelle an sich oder um einen unbekannten Factor in einer Celle, der von dieser nicht zu scheiden ist.

Wird bei Tumoren von Virulenz gesprochen, so darf dieser Begriff nicht dem der Pathogenität gleichgesetzt werden, aber ebensowenig mit einer einzelnen bestimmten Eigenschaft des Virus oder der Tumorcelle, wie ,,Toxinbildung" oder Eindringungskraft oder anderer besonders auffallender Characteristica des Krankheitserregers.

Eine experimentelle Feststellung der Tumorvirulenz ist nur in einem Teil der Fälle möglich: bei den ausgesprochenen Virustumoren und bei den in Ascitesform sich entwickelnden Tumoren, die in den letzten Jahren immer mehr in den Vordergrund des Interesses kommen. Bei diesen beiden Kategorien von Tumoren ist eine exacte quantitative Messung der Virulenz möglich.

An dieser Stelle soll nur über die Virulenz von Ascitestumoren gesprochen werden, da die hier angestellten Versuche ursprünglich bestimmt waren, eine nähere Einsicht über die Entwicklungsmöglichkeit von Gelbfiebervirus mit den Cellen des KREBS-2-Ascitescarcinoms zu gewinnen.

Um sich ein eindrucksvolles Bild von der Virulenz zu machen,

muss man eine in der Zeit verlaufende Kurve betrachten. Hierbei brauchen die zahlreichen Factoren der beiden Parteien nicht näher analysiert zu werden.

Um dies bei Virusinfectionen (und anderen Infectionen) deutlich zu machen, benutzten COLLIER, DE ROEVER-BONNET und HOEKSTRA (1959) Versuche mit Gelbfieberinfection der weissen Maus. Sie gingen von Versuchen aus, in denen Versuchstiere einer möglichst gleichmässig zusammengesetzten Population mit fallenden Verdünnungen einer ebenfalls möglichst gleichmässigen Erregerpopulation so inficiert werden, dass (möglichst) in den niedrigen Verdünnungsgraden alle Tiere erkranken oder sterben und in den höchsten Verdünnungsgraden (möglichst) alle Versuchstiere am Leben bleiben. Dann wird von dem täglichen Status des Gesamtversuches die LD_{50} nach REED und MUNCH (1938) berechnet.

TECHNIK.

Mäusestamm: Die Versuche wurden mit dem Swiss-Stamm des Instituts durchgeführt. Die Tiere waren 8–10 Wochen alt.

Tumorstamm: Für alle Versuche wurde der KREBS-2-Ascitescarcinom-Stamm gebraucht, der uns freundlicherweise durch Herrn Prof. Dr. O. MÜHLBOCK (Antonie van Leeuwenhoekhuis, Amsterdam) zur Verfügung gestellt wurde.

Infectionstechnik: Von intraperitoneal inficierten Tieren wurde durch Punktion Ascites gewonnen. Hiervon wurden in Zehnerpotenzen Verdünnungsreihen angefertigt, wobei als Verdünnungsflüssigkeit 1%iges Rinderalbumin des Bluttransfusionsdienstes benutzt wurde. Die Mäuse wurden hiermit bei intraperitonealer oder subcutaner Application mit je 0,2 ml inficiert, bei intracerebraler Application unter Aethernarcose mit je 0,02 ml. In der Regel wurden die Verdünnungen von $^1/_1$, $^1/_{10}$ . . . bis $^1/_{1\,000\,000}$ benutzt, wobei in jeder Gruppe 5–6 Mäuse inficiert wurden. Gelegentlich wurde mit der Verdünnung von $^1/_{10}$ begonnen oder der Verdünnung von $^1/_{100\,000}$ geendigt. Die inficierten Tiere werden beobachtet, bis keine neuen Erkrankungs- oder Todesfälle mehr vorkommen. Nach intracerebraler Infection ist dieser Zeitpunkt ziemlich schnell erreicht, und nach 30 Tagen kommt es kaum noch zu Todesfällen. Nach intraperitonealer Impfung verläuft die Infection deutlich langsamer, und nach subcutaner kann eine oder die andere Maus ausserordentlich lange am Leben bleiben.

TABELLE 1.

Intracerebrale Infection mit 0,02 ml Ascites (40 Tage alt) in fallenden Dosen.

Dosis	1/1						1/10						1/100						1/1000						1/10 000						1/100 000						1/1 000 000					
Tier-No.:	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1. Tag	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
2. ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
3. ,,	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
4. ,,	†	k	k	k	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
5. ,,		†	†	†	†	k	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
6. ,,						k	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
7. ,,						†	†	k	k	k	k	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
8. ,,								†	k	k	k	k	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
9. ,,									†	†	†	k	k	k	k	k	,,	,,	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
10. ,,												†	†	k	k	k	,,	,,	k	,,	,,	,,	,,	,,	,,	,,	,,	,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
11. ,,														†	†	k	k	k	k	k	k	k	,,	,,	k	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
12. ,,																†	k	k	†	†	k	k	k	k	k	k	k	,,	,,	,,	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
13. ,,																	k	k			†	k	k	k	k	k	k	,,	,,	,,	k	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
14. ,,																	k	k				†	k	k	†	k	k	k	k	,,	†	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
15. ,,																	†	†					†	†		†	k	k	k	k		,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
16. ,,																											†	k	k	k		,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
17. ,,																												†	†	†		,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
18. ,,																																,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
19. ,,																																,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
35. ,,																																,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,

m = munter; k = krank; † = tot.

B e r e c h n u n g : Von den an jedem Tage toten Mäusen wird nun die LD_{50} nach Reed und Munch (1938) berechnet. Hierbei werden nicht die täglich beobachteten neuen Todesfälle der einzelnen Gruppen als Ausgangspunkt benutzt, sondern die cumulierten Todesfälle der Gruppen bis zu diesem Tage.

Die erhaltenen Werte werden auf „ml" umgerechnet, indem bei der intracerebralen Infectionsdosis von 0,02 ml die gefundenen Werte mit 50 und bei der subcutanen oder intraperitonealen Infectionsdosis von 0,2 ml die gefundenen Werte mit 5 multipliciert werden. Auf diese Weise lassen sich alle Versuche miteinander vergleichen. Bei der graphischen Darstellung werden auf der Abscisse die Tage und auf der Ordinate die Logarithmen der reziproken Werte der LD_{50} eingetragen.

Besprechung der Ergebnisse.

I n t r a c e r e b r a l e I n f e c t i o n : Hier ist der Verlauf des Versuches am eindruckvollsten. Schon nach wenigen Tagen weisen die mit der stärksten Dosis inficierten Mäuse sehr deutliche Krankheitserscheinungen auf und gehen sehr schnell zugrunde. Bei den schwächeren Infectionsdosen ist die Incubationszeit verlängert, auch das manifeste Krankheitsstadium kann verlängert sein. In Tabelle 1 ist der Verlauf eines derartigen Versuches beschrieben, und zwar ist für jede Maus besonders der Tag von Erkrankung und der Todestag angegeben. In diesem Fall kann also die Virulenzcurve auf zwei verschiedene Weisen ausgearbeitet werden: Einerseits vom Tage der Erkrankung und andererseits vom Todestage ausgehend. Im ersten Falle ist aus der Curve die Incubationsperiode zu ersehen, im anderen Fall die Incubationsperiode + die Dauer des manifesten Krankheitsverlaufes.

Die in der Tabelle 1 gesammelten Werte müssen nun zusammengezogen werden, um die weitere Berechnung durchzuführen. In Tabelle 2 ist dies für die Sterblichkeit durchgeführt. In der vorletzten Kolomme sind die Werte für LD_{50}/ml ausgerechnet, und in der letzten Kolomme finden sich hierzu die Logarithmen der reziproken Werte. In genau der gleichen Weise lassen sich auch die Werte für die Erkrankungstage ausrechnen.

Die beiden Curven, die sich aus den so erhaltenen Werten entwickeln lassen, sind in Figur 1 wiedergegeben. Während des Ansteigens der beiden Curven lässt sich aus dem horizontalen Abstand

TABELLE 2.

Cumulative Sterblichkeit von Mäusen nach Infection mit fallenden Dosen von Ascites (40 Tage alt) cerebral (nach Tabelle 1).

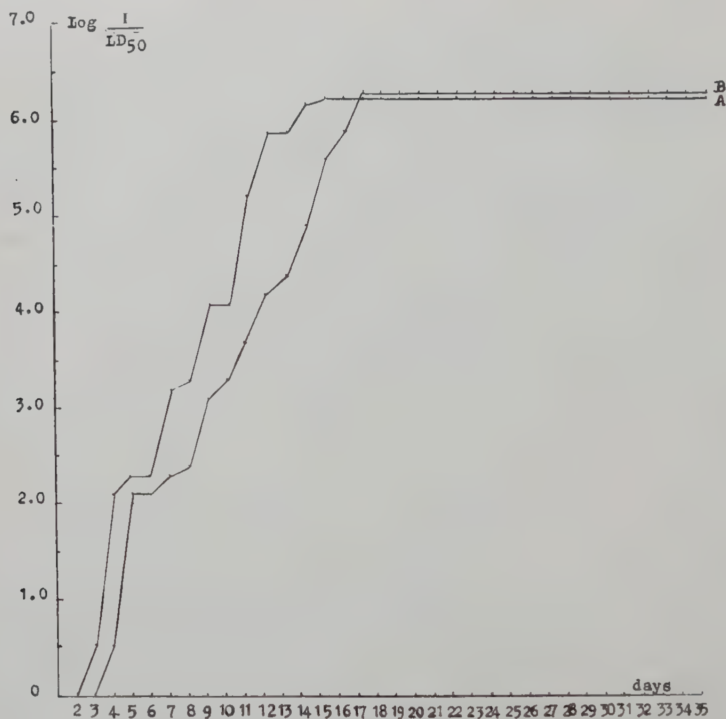
	Dosis 0,02 ml von der Verdünnung von:							LD_{50}/ml	$\frac{1}{\log}$
	$^1/_1$	$^1/_{10}$	$^1/_{100}$	$^1/_{1000}$	$^1/_{10.000}$	$^1/_{100.000}$	$^1/_{1000.000}$		
1.Tag	$^0/_6$	$^0/_6$	$^0/_6$	$^0/_6$	$^0/_6$	$^0/_6$	$^0/_6$	0	
2. ,,	,,	,,	,,	,,	,,	,,	,,	0	
3. ,,	,,	,,	,,	,,	,,	,,	,,	0	
4. ,,	$^1/_6$	,,	,,	,,	,,	,,	,,	$> {}^1/_{50}$	<1
5. ,,	$^5/_6$	,,	,,	,,	,,	,,	,,	$^1/_{125}$	2,1
6. ,,	,,	,,	,,	,,	,,	,,	,,	,,	2,1
7. ,,	$^6/_6$	$^1/_6$	,,	,,	,,	,,	,,	$^1/_{200}$	2,3
8. ,,	,,	$^2/_6$	,,	,,	,,	,,	,,	$^1/_{280}$	2,4
9. ,,	,,	$^5/_6$	,,	,,	,,	,,	,,	$^1/_{1250}$	3,1
10. ,,	,,	$^6/_6$	$^1/_6$	,,	,,	,,	,,	$^1/_{2000}$	3,3
11. ,,	,,	,,	$^3/_6$	,,	,,	,,	,,	$^1/_{5000}$	3,7
12. ,,	,,	,,	$^4/_6$	$^2/_6$	,,	,,	,,	$^1/_{16000}$	4,2
13. ,,	,,	,,	,,	$^3/_6$	,,	,,	,,	$^1/_{24500}$	4,4
14. ,,	,,	,,	,,	$^4/_6$	$^1/_6$	$^1/_6$	,,	$^1/_{87500}$	4,9
15. ,,	,,	,,	$^6/_6$	$^6/_6$	$^2/_6$	,,	,,	$^1/_{370000}$	5,6
16. ,,	,,	,,	,,	,,	$^3/_6$	,,	,,	$^1/_{700000}$	5,9
17. ,,	,,	,,	,,	,,	$^6/_6$	,,	,,	$^1/_{2000000}$	6,3
18. ,,	,,	,,	,,	,,	,,	,,	,,	,,	6,3
19. ,,	,,	,,	,,	,,	,,	,,	,,	,,	6,3
35. ,,	,,	,,	,,	,,	,,	,,	,,	,,	6,3

beider deutlich die Länge des manifesten Krankheitsverlaufes ablesen, die etwas zunimmt, wenn die Incubationszeit länger wird. Wenn kein weiteres Ansteigen mehr zu erkennen ist, wenn also das Maximum erreicht ist, decken sich beide Kurven.

In der Praxis hat sich ergeben, dass die aus den cumulativen Todesfällen entstehende Kurve in der Regel vollkommen ausreichend ist, um die Virulenz deutlich zu characterisieren.

In Figur 2 sind vier verschiedene Virulenz-Titrierungen zusammengestellt, die von verschieden altem Ascites durchgeführt sind. Es zeigt sich hierbei kein sehr deutlicher Unterschied bei den Kurven, die sich aus 5, 6 und 12 Tage altem Ascites entwickeln. Demgegenüber ist deutlich zu ersehen, dass die Virulenz des 36 Tage alten Ascites wesentlich geringer ist. Die Curve beginnt später und weist ein viel geringeres Maximum auf als die drei anderen Curven.

Intraperitoneale Infection: Während nach in-

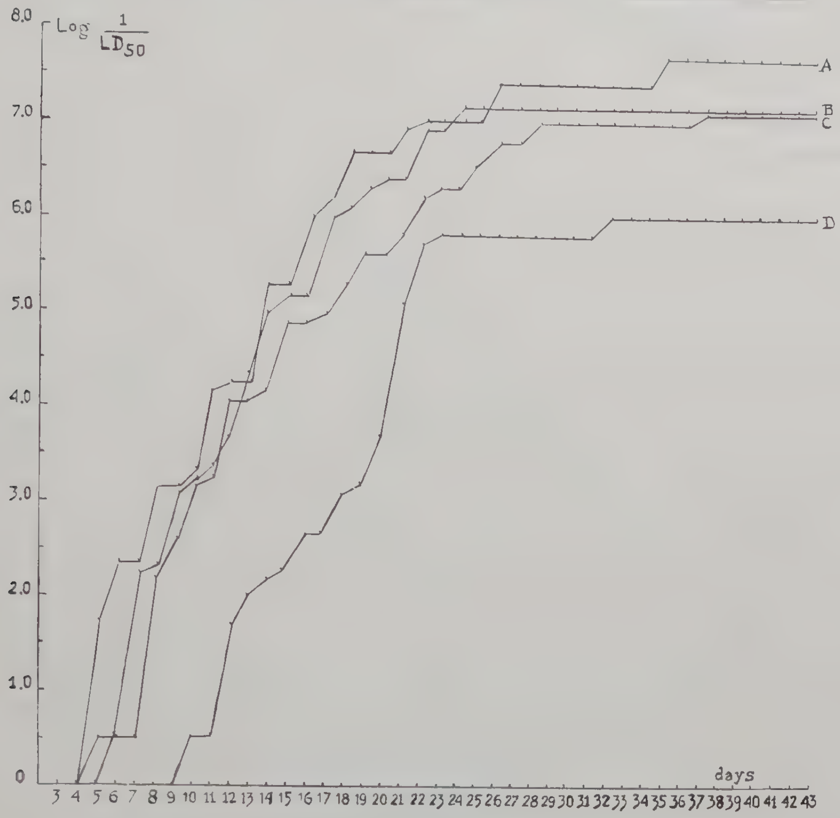


Figur 1. Infection intracerebral mit 0,02 ml Ascites von 40 Tagen: Virulenzcurven von Tabelle 1 und 2.
A = cumulative Erkrankungen = KD_{50}
B = cumulative Todesfälle = LD_{50}

tracerebraler Infection mit Tumorascites der sehr foudroyante Verlauf der Erkrankung deutlich durch den steilen Anstieg der Virulenzcurve characterisiert wird, ist nach intraperitonealer Infection ein viel langsamerer Anstieg zu verzeichnen.

In Figur 3 finden sich vier Virulenzcurven zusammengestellt, die sich nach intraperitonealer Infection mit verschieden altem Ascites entwickelt haben. Der Unterschied zwischen 6 und 12 Tage altem Ascites ist nicht sehr gross, der 18 Tage alte ist schon deutlich weniger virulent, und der 36 Tage alte weist die niedrigste Virulenz auf.

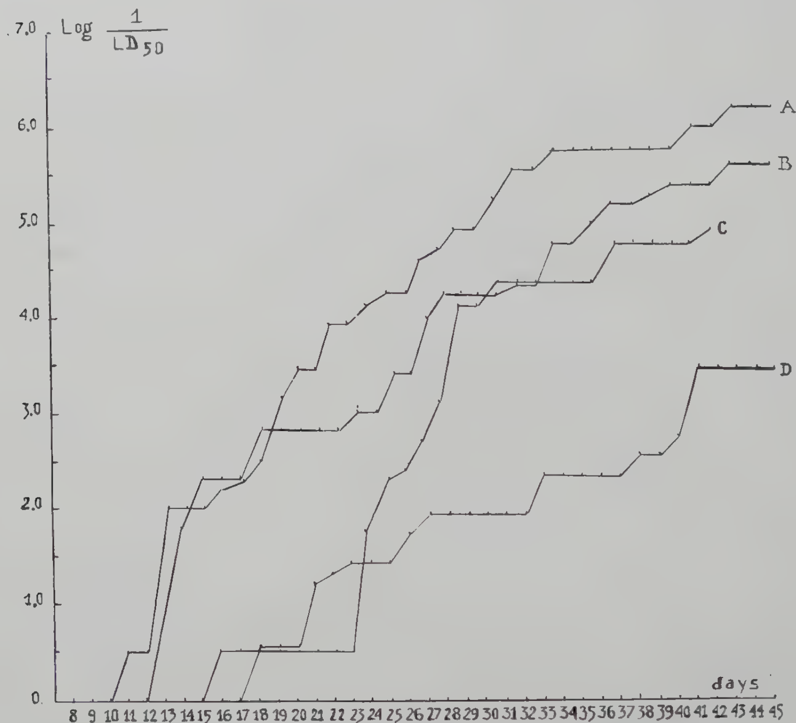
Porte d'entrée: Wie stark die Virulenz von Ascitestumoren von der Applicationsweise abhängt, geht deutlich aus der folgenden Figur 4 hervor. Hier sind die Virulenzcurven von 4 Tage altem



Figur 2. Intracerebrale Infection mit 0,02 ml Ascites
A = Ascites 5 Tage alt
B = Ascites 6 Tage alt
C = Ascites 12 Tage alt
D = Ascites 36 Tage alt.

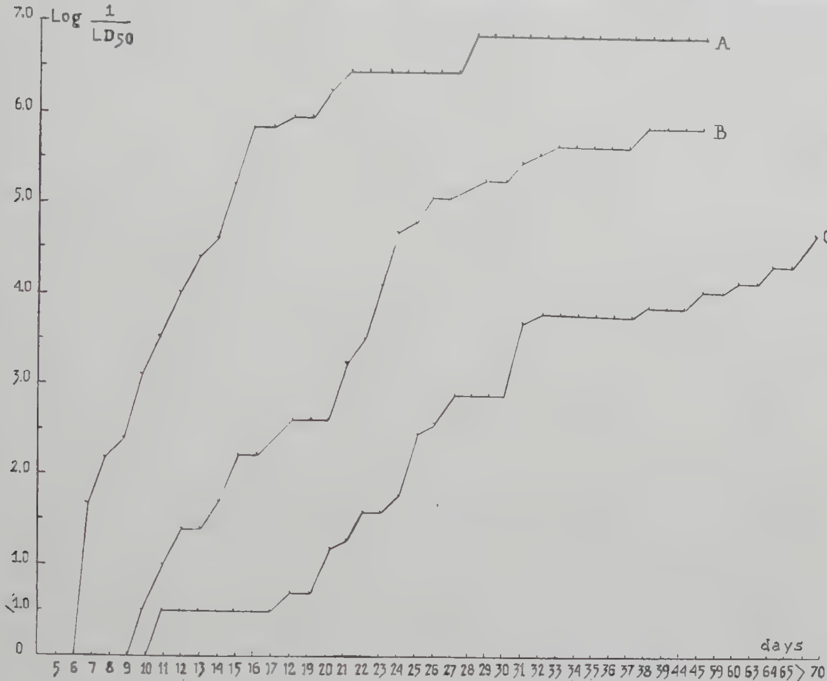
Ascites bei intracerebraler, intraperitonealer und subcutaner Verimpfung einander gegenüber gestellt. Die höchste Virulenz zeigt sich bei intracerebraler Verimpfung des Materials, die niedrigste bei subcutaner. Dazwischen liegt die Virulenz bei intraperitonealer Infection. Bei intracerebraler Infection ist das Maximum am 28. Tage erreicht, bei intraperitonealer am 38. Tage und bei subcutaner erst nach dem 70. Tage.

A u f b e w a h r t e r A s c i t e s : Schon seit den ersten Versuchen von LOEWENTHAL und JAHN (1932) und COLLIER und JAHN (1934) ist bekannt, dass Tumorascites bei Eisschrank-Temperatur



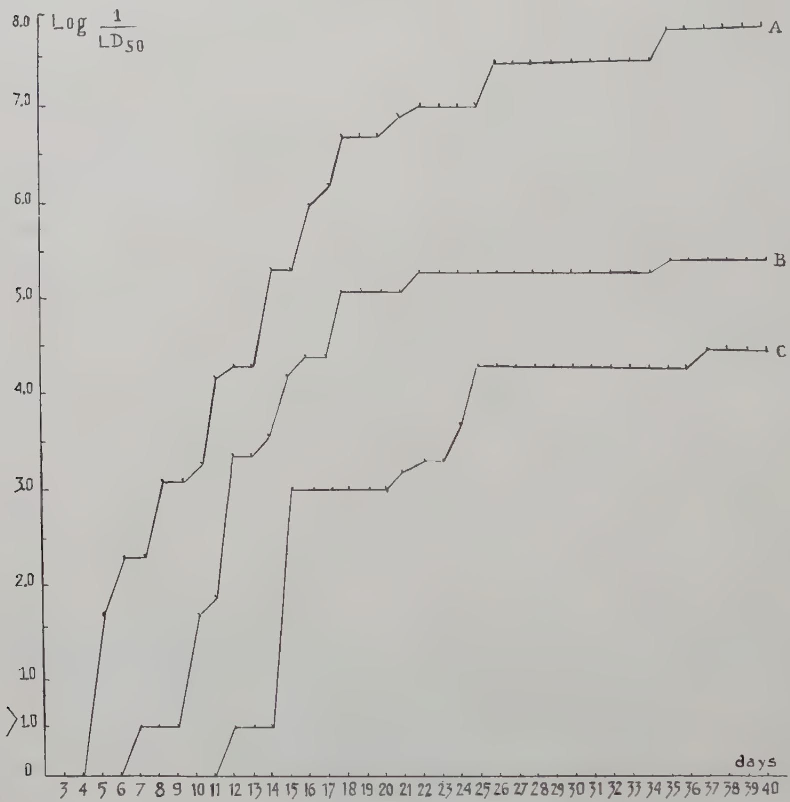
Figur 3. Intraperitoneale Infection mit 0,2 ml Ascites
A = Ascites 6 Tage alt
B = Ascites 12 Tage alt
C = Ascites 18 Tage alt
D = Ascites 36 Tage alt.

gut aufbewahrt werden kann. Und in der Tat ist es möglich, durch Verimpfung von unverdünntem Ascites aus dem Eisschrank noch nach relativ langer Zeit wieder Ascitestumoren zu erzeugen. Durch Virulenz-Titrierungen ist es aber möglich nachzuweisen, dass im Eisschrank die Virulenz des aufbewahrten Tumorascites herabgesetzt wird. In Figur 5 ist 5 Tage alter Ascites austitriert und zwar direct nach Entnahme aus dem Peritoneum, nach 4 und nach 7 Tagen Aufbewahren im Eisschrank bei $\pm$ 4°C. im unverdünnten Zustand. Die Titrierung erfolgte auf die empfindlichste Weise, nämlich intracerebral. Aus diesen Virulenzcurven ist deutlich zu ersehen, dass das frische Material erheblich virulenter ist als das im Eisschrank aufbewahrte; es übertrifft das 4 Tage alte mit einem Maximum von log 2,4 und das 7 Tage alte Material mit einem solchen von log 3,3!



Figur 4. Infection mit 4 Tage altem Ascites
A = intracerebral 0,02 ml
B = intraperitoneal 0,2 ml
C = subcutan 0,2 ml.

Cytostatische Inhibitoren: In Figur 2 und 3 hatte sich gezeigt, dass sehr alter Ascites deutlich eine geringere Virulenz aufwies, als frischer Tumorascites. Hierbei scheinen sich entwickelnde Inhibitoren eine gewisse Rolle zu spielen, durch welche die beobachtete Virulenzverminderung bedingt oder doch unterstützt wird. In diese Richtung spricht folgender Versuch: Von einer Maus, die vor 43 Tagen mit Ascitestumor und Gelbfiebervirus zusammen inficiert worden war, wurde Ascites abgenommen, bei 5000 Umdrehungen 10 Minuten lang centrifugiert und 20 Minuten lang bei 56°C. inactiviert. Frischer Ascites wurde nunmehr mit diesem Ascites so verdünnt, dass Endconcentrationen von $^1/_{10}$ bis $^1/_{100\,000}$ entstanden. Als Controllen wurden die gleichen Verdünnungen mit 1%igem Rinderalbumin hergestellt. Beide Verdünnungsserien wurden 24 Stunden in den Eisschrank zum Binden gebracht und am folgenden Tage auf Mäuse verimpft. Das Ergebnis dieses Versuches ist in Figur 6 zusammengestellt.

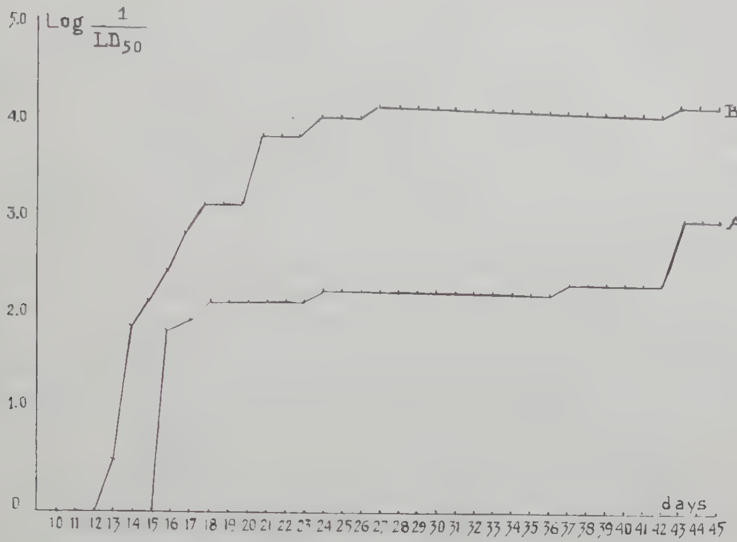


Figur 5. Einfluss des Aufbewahrens von 5 Tage altem Ascites bei ± 4 °C.
A = intracerebral 0,02 ml direct verimpft
B = intracerebral 0,02 ml nach 4 Tagen bei ± 4°
C = intracerebral 0,02 ml nach 7 Tagen bei ± 4°.

Es zeigt sich hier deutlich, dass die Virulenzcurve des mit altem Ascites verdünntem Ascitesmaterials viel niedriger verläuft als die, welche mit Rinderalbumin verdünnt worden ist. Eine lange Zeit hindurch beträgt der Abstand beider Curven log 1,7–1,9 und nach Erreichen des Maximums immer noch log 1,3. Dass nach einem Krankheitsverlauf von 43 Tagen cytotoxische Stoffe gegen die Tumorcellen entstanden sind, ist nicht ohne Weiteres abzulehnen, offen bleibt indessen noch, ob es sich um Antistoffe oder andere Inhibitoren handelt.

Discussion.

Bei den Virulenzcurven sind drei Merkmale zu erkennen, nämlich



Figur 6. Cytotoxischer Einfluss von 43 Tage altem Ascites einer Maus, die gleichzeitig mit Gelbfiebervirus (Stamm 17D) inficiert war, bei intraperitonealer Infection mit 0,2 ml.

A = Ascites verdünnt in toxischem centrifugierten Ascites.

B = Ascites in 1% igem Rinderalbumin verdünnt.

die Gesamt-Krankheitsdauer, die Verlauf- oder Anstiegschnellheit und die absolute Höhe der Virulenz. Alle diese drei Merkmale werden sowohl durch die Factoren der Pathogenität des Tumorerregers, der Tumorcelle, als auch durch die Empfänglichkeit und Resistenz des Wirtes beeinflusst. Dies drückt sich deutlich in den entstehenden Curven aus.

Eine andere Betrachtungsweise besteht darin, dass man nicht von den Todesfällen, sondern vom Beginn der manifesten Erkrankung der Versuchstiere ausgeht. In diesem Fall stellt das erste Merkmal nicht den Gesamt-Krankheitsverlauf dar, sondern die Incubationsperiode. Nach intracerebraler Infection zeigt sich unverkennbar der Beginn von Gehirnerscheinungen, nach intraperitonealer Infection weist die im Vergleich zu den Controlltieren deutliche Gewichtszunahme auf den Beginn der Erkrankung, und bei subcutaner Verimpfung des Tumormaterials lässt sich der Beginn der Geschwulstbildung durch Abtasten feststellen.

In den hier mitgeteilten Versuchen lässt sich an Hand von den Virulenzcurven zeigen, dass die intracerebrale Verimpfung von

Ascitestumor-Material wohl die empfindlichste Methode darstellt, während die intraperitoneale Verimpfung eine geringere Virulenz ergibt, und die subcutane Verimpfung eine noch geringere. Aus hier nicht näher mitgeteilten Versuchen scheint hervorzugehen, dass bei dem so ausserordentlich schnell verlaufenden intracerebralen Krankheitsprocess durch Immunstoffe oder Cytostatica keinerlei Einfluss zu verzeichnen ist.

Weiterhin aber geht aus den hier mitgeteilten Versuchen hervor, dass das Alter des verimpften Tumorascites nicht ohne Einfluss auf den Verlauf der Virulenzcurven ist. Je älter der benutzte Ascites, desto niedriger ist die Virulenz bei den Mäusen. Bei allen Versuchen mit Tumorascites ist also auf das Alter des Materials zu achten. Bei sehr altem Ascites ist jedenfalls auch damit zu rechnen, dass sich im Laufe der Zeit cytotoxische Stoffe gegen die Tumorcellen entwickelt haben. Dergleichen cytotoxische Stoffe sind mittels der hier angewandten Methodik ziemlich gut nachzuweisen.

Schliesslich konnte in den hier mitgeteilten Versuchen nachgewiesen werden, dass das Aufbewahren von Tumorascites im Eisschrank nicht ohne Einfluss auf die Virulenz ist. Bei einer Verimpfung von konserviertem Ascites im unverdünnten Zustande wird man hiervon nichts merken, da die Infectionsdosis in diesem Fall ja ausserordentlich gross ist. Anders ist es indessen bei der serienweisen Verimpfung fallender Verdünnungen. Hier zeigt sich bereits nach einem Aufenthalt von 4 Tagen bei $\pm$ 4°C. ein unverkennbarer Virulenzverlust bei der so überaus empfindlichen cerebralen Verimpfung.

Bei den Versuchen mit Ascitestumoren kann es – besonders bei der Verimpfung von höheren Verdünnungen – vorkommen, dass die Tiere keinen Ascites bilden, wohl aber mit soliden Tumoren zugrunde gehen. Bei der Virulenzcurven-Methode fallen solche Tiere nicht als ,,Nicht-Ascitesbildner'' oder als ,,solide-Tumorbildner'' aus. Sie üben deutlich ihren Einfluss auf die Virulenzcurve aus, indem sie nach relativ langer Zeit die absolute Virulenz ansteigen lassen. Schliesslich kommt es bei den Versuchen mit Ascitestumoren nicht auf die Ascitesbildung, sondern auf die Tumorentwicklung an.

Nach Klein (1950a) und Klein, Kurnick und Klein (1950) bestehen verschiedene Criteria für die Entwicklung von Ascitestumoren, so die Ueberlebenszeit, das Körpergewicht, der Prozentsatz des Angehens der Infection als Function der Grösse des Inoculums, der Procentsatz und die Concentration freier Tumorcellen im Ascites, die Mitoserate usw.

Auf die Bedeutung der Ueberlebenszeit hatten bereits HAAGEN und SEEGER (1938) hingewiesen. Nach LETTRÉ (1941a, 1941b, 1950) und KLEIN (1950b) ist sie als das Characteristicum eines bestimmten Stammes anzusehen. Genauere Ziffer über den Einfluss der verimpften Zellzahlen auf die Ueberlebensdauer geben u.a. KLEIN, KURNICK und KLEIN (1950), LETTRÉ (1950), und KLEIN und KLEIN (1951) an. GOLDBERG, KLEIN und KLEIN (1950) wiesen auf die Abhängigkeit der LD_{50} von der Zahl der verimpften Cellen beim EHRLICHschen Ascitescarcinom hin. Demgegenüber ist nach SCHÜMMELFEDER, MENGES und WESSEL (1956) beim MCal-Ascitestumor die Ueberlebenszeit in den Grenzen von 17×10^6 bis 39×10^6 verimpften Cellen unabhängig von der verimpften Cellenanzahl.

Eine graphische Methode, um die Entwicklung verschiedener Ascitesmuster zu vergleichen, wird durch KLEIN (1950a) angegeben. KLEIN, KURNICK und KLEIN (1950) wiesen auch darauf hin, dass diese Curve um so flacher wurde, je weniger Cellen verimpft wurden, und dass die Todesfälle auch desto später beginnen.

Werden aber die Infectionen mit Cellzahlen durchgeführt, die kleiner sind als das Minimum, durch welches in 100 % Ascitesbildung verursacht wird, so ist nach KLEIN (1950a) die Lebenszeit deutlich verlängert. Die Tiere, welche keinen Ascites gebildet haben, gehen schliesslich mit soliden Tumoren im Peritoneum zugrunde.

Beim KREBS-2-Ascitescarcinom können nach PATT und BLACKLOCK (1954b) die Differenzen in der Ueberlebenszeit beträchtlich grösser sein als die Zeit, die nötig ist, die Zellzahlen entsprechend zu vergrössern. So kann am 6. Tage ein LD_{50} bei einem Inoculum von 10^7 Cellen erreicht werden, am 17. Tage mit einem Inoculum von 10^6 Cellen, während die entsprechende Cell-Wachstumscurve nur durch 2,3 Tage getrennt ist. Hierbei spielt vielleicht eine Rolle, dass sich der Wirt bei einer längeren Verlaufszeit besser an den Stress anpassen kann.

Nach KLEIN (1950a) lässt sich auf Basis der Ueberlebenszeit leicht der Einfluss eines Chemotherapeutikums berechnen, worauf später auch CREECH, HAUSCHKA, HANKWITZ, LITTLETON und ANDRE (1955), FURST, CUTTING und GROSS (1955) und HORN (1955) hinwiesen.

Nach LETTRÉ (1941a, 1941b, 1950) ist die Ascitesbildung mit der Vermehrung der Geschwulstcellen funktionell verknüpft. Das Gewicht der Ascitesflüssigkeit ist für den Character der Gewichtscurve der Maus bestimmend, und daher ist bei intraperitonealer Application der Tumorcellen das Körpergewicht der Tiere ein hinreichend zuverlässiger Massstab für die Ascitesbildung und das Wachstum des Ascitestumors. Der Grad der Gewichtszunahme ist auch als Massstab für eine eventuelle Entwicklungshemmung zu beurteilen. Aehnlich äussern sich PATT und BLACKLOCK (1954a) und LEVIN, STERN, EKSTEIN und WOIDOWSKY (1953). Allerdings kann Gewichtsveränderung auch durch andere Factoren bedingt sein, was LETTRÉ (1950) selber zugibt und KLEIN (1950a) betont. PATT und BLACKLOCK (1954a) heben ebenfalls hervor, dass Gewichtsveränderungen auch durch Anasarka und durch „wasting" entstehen kann.

Später berichtet KLEIN (1956), dass bei den EHRLICH- und KREBS-2-Ascitestumoren während des grössten Teils der Wachstumperiode mit Aus-

nahme des Terminalstadiums das Postulat von LETTRÉ zu Recht besteht. Dies gilt aber nicht für alle Ascitestumoren, und auch dann nicht, wenn die Cellvermehrung gehemmt wird, wie auch RÉVÉSZ (1955) betonte. Aehnliche Ansichten drückte GOLDIE (1956) aus. Nach SCHÜMMELFEDER, MENGES und WESSEL (1956) kann bei dem MCal-Ascitestumor die Streuung der Gewichtswerte der einzelnen Tiere zwischen +21,5 g und —2,5 g liegen!

Auch SUGIURA (1953) weist auf die relativ grossen individuellen Schwankungen bezüglich der Tumorenempfindlichkeit hin, indem manche Tiere nur wenig an Gewicht zunehmen und andere selbst überhaupt keinen Ascites bilden. Er beurteilt die Ergebnisse nach dem Grade der Schwellung des Abdomens, was mit dem Volumen bzw. Gewicht der Ascitesmenge in Verband gebracht werden kann.

Die Menge der verimpften Cellen bedingt das Angehen der Ascitestumoren. Nach KLEIN (1950a) und KLEIN, KURNICK und KLEIN (1950) geht beim Ueberschreiten einer bestimmten Cellenanzahl die Infection in 100 % an. Beim EHRLICH-Ascitescarcinom liegt die kleinste Anzahl Cellen, die noch bei 100 % der Tiere Ascites erzeugt, bei 800.000. SUGIURA und CREECH (1956) verimpfen in der Regel ± 1 Million Cellen in 0,1 ml.

Wenn LETTRÉ (1950) die Dosis auf 1 Million verminderte, hatte er doch stets 100% Impferfolg, wenn auch die Entwicklung verlangsamt war und der Tod der Tiere nach 27-46 Tagen eintrat.

Nach KLEIN, KURNICK und KLEIN (1950) hängt beim EHRLICH-Ascitescarcinom innerhalb der Grenzen von 1×10^5 bis 21×10^6 verimpfter Cellen die Ueberlebensdauer von dieser verimpften Menge ab, bei höheren Cellzahlen ist sie davon unabhängig. RUBNER (1958) erzielt eine gute Erfolgsquote durch 300.000 Cellen intraperitoneal oder 30-100.000 Cellen intramusculär.

HAUSCHKA (1953a) erhielt bei Ein-Cell-Verimpfungen nach 18-23 Tagen in 4 % der Fälle Ascitesbildung.

Ueber die totale Menge von Tumorcellen im Ascites äusserten sich u.a. HAAGEN und SEEGER (1938), GOLDBERG, KLEIN und KLEIN (1950), KLEIN und KLEIN (1951), SUGIURA (1953), KLEIN (1956), SCHÜMMELFEDER, MENGES und WESSEL (1956) und SUGIURA und CREECH (1956).

Ueber die Technik der Cellzählung berichteten u.a. KLEIN und RÉVÉSZ (1953), PATT, BLACKLOCK und DRALLMEIER (1953), SCHLEICH und WRBA (1953), BATHER (1954), LUCKE und BERWICK (1954), PATT und BLACKLOCK (1954b), RÉVÉSZ und KLEIN (1954) und KLEIN (1955, 1956). Ueber die Technik der Feststellung der Lebensfähigkeit der Tumorcellen vergl. KLEIN (1950). Schon frühzeitig hatten HAAGEN und SEEGER (1938) auf die Wichtigkeit hingewiesen, die Anzahl der lebenden und toten Cellen auseinander zu halten. Und in der Tat finden sich nach KLEIN (1956) hier sehr grosse individuelle Unterschiede, abhängig von Celldegeneration und Celltod während des Wachstumscyclus. Nach der Stammcellen-Theorie von HAUSCHKA (1953b) ist weiterhin nur ein Teil der Cellen imstande, normal und regelmässig Mitosen zu bilden, sodass sich im Tumorascites neben vermehrungsfähigen auch zahlreiche nicht-vermehrungsfähige Cellen finden.

Das Cellwachstum im Tumorascites hängt nach GOLDIE (1956) vom Gleichgewicht zwischen Flüssigkeitsdrainage und Durchlässigkeit ab, Factoren,

die beide schwanken können. Verschiedene Agentien, welche die Permeabilität der Capillaren beeinflussen (wie Cortison) oder welche die Durchlässigkeit duch auftretende Haemorrhagien beeinflussen (wie Heparin) haben merklichen Einfluss auf die Schwankungen der Ascitesflüssigkeit und des Gehaltes an Tumorcellen. Durch Verschluss der Lymphgefässe oder Bluttgefässe, die sogar durch wachsende Tumorcellen verstopft werden können, vermag das Volumen des Ascites unverhältnismässig schnell zuzunehmen, wobei dann eine Abnahme der Cellen vorgetäuscht werden kann. Ferner muss man auch bedenken, dass die Cellen unter dem Einfluss von Trauma der Serosa, Fibrinniederschlägen usw. aus der Flüssigkeit verschwinden können.

Schliesslich ist auch die Teilungsgeschwindigkeit der Cellen nicht immer gleich. So wiesen PATT und BLACKLOCK (1954a) darauf hin, dass sich die Tumorcellen des KREBS-2-Ascitescarcinoms zuerst einige Tage lang exponentiell vermehren, worauf die Cellteilung verlangsamt wird bis zu einer Asymptote von etwa 10^9. Beim EHRLICH-Ascitescarcinom schwankt nach KLEIN (1950a) die Cellzahl um 25,5 %, wozu noch der Irrtum beim Zählen (6,8 %) kommt.

Nach KLEIN (1956) lässt sich das Wachstum von Ascitestumoren erheblich besser verfolgen als das solider Tumoren. Und doch ergeben sich auch hier noch grosse Schwierigkeiten, da Wachstum und Cellvermehrung sich nicht auf den Ascites beschränken. In mehr oder weniger hohem Grade kommt es auch zu Infiltrationen solider Gewebe durch Tumorcellen. Die Gesamtzahl freier Tumorcellen stellt nur die Majorität dar. Im Beginn des Verlaufes ist nach KLEIN (1950a), KLEIN und RÉVÉSZ (1953), LETTRÉ (1950), BAILIF (1954), RÉVÉSZ und KLEIN (1954), PATT und STRAUBE (1956) das Wachstum wahrscheinlich schnell genug, um nicht ohne Weiteres solide Herde zu bilden. Wenn sich dann aber im Verlaufe der Zeit doch Cellen an der Oberfläche des Peritoneums festheften und weiter entwickeln, ist der Zusammenhang zwischen Ascitesmenge und Cellenzahl gestört.

Schliesslich weisen PATT und STRAUBE (1956) darauf hin, dass nach der Beginnphase activen Wachstums eine Periode progressiv abnehmenden Wachstums folgt. Die Cellen zeigen gesteigerte Concurrenz für die verschiedenen Nährstoffe. Der Wirt kann nicht mehr alles liefern wie beim Beginn des Krankheitsprocesses. Es bilden sich toxische Stoffwechselproducte. Der Wirt beginnt Krankheitserscheinungen zu zeigen.

Bei der Characterisierung der Virulenz bei Tumoren muss naturgemäss auch die Metastasenbildung und der Grad des infiltrativen Wachstums mit einbezogen werden, ebenso bei den in Ascitesform wachsenden Tumoren die Entwicklung massiver Cellmassen an der Einstichstelle, im Peritoneum usw. Dies alles ist durch Wägen und Messen nicht festzustellen, hat aber zweifellos Einfluss auf die Lebensdauer des Wirtes.

Eine Verzögerung im Angehen der Infection deutet nicht immer auf eine Verminderung der Virulenz an sich hin, kann vielmehr auch auf Verminderung der verimpften lebensfähigen Cellen zurück-

zuführen sein. In so einem Fall ist aber bei Virulenzcurven sowohl die Anstiegschnellheit als auch die absolute Höhe der Virulenz unverändert geblieben. Nur die Incubationsperiode ist verlängert.

Ein wirklicher Virulenzverlust liegt vor, wenn nach SUGIURA (1953) nach 5 Tage Aufbewahren des Ascites bei 3–4°C. weniger Mäuse Ascites ausbilden. In diesem Fall zeigen auch die hier mitgeteilten Virulenzcurven deutlich, dass Aufbewahren im Eisschrank Virulenzverlust zur Folge hat. Auch die Behauptung von RUBNER (1958), dass die Virulenz der Cellen im Durchschnitt geringer wird, je älter der Ascites ist, lässt sich an Hand der hier mitgeteilten Curven als durchaus richtig gesehen feststellen.

Zusammenfassung.

Es wird eine Definition für die Virulenz von den in Ascitesform wachsenden Tumoren gegeben und danach eine Methode mitgeteilt, nach der es möglich ist, diese Virulenz graphisch als „Virulenzcurve" darzustellen.

Mit Hilfe dieser Virulenzcurven lässt sich nachweisen, dass sowohl bei intracerebraler als auch bei intraperitonealer Verimpfung die Virulenz abnimmt je älter der verimpfte Ascites ist. Weiterhin ist die Virulenz abhängig von der porte d'entrée, indem sie bei intracerebraler Infection maximal hoch ist, bei intraperitonealer mässig hoch und bei subcutaner relativ niedrig.

Die Virulenz nimmt beim Aufbewahren des unverdünnten Ascites im Eisschrank bereits nach 4 Tagen deutlich ab.

In sehr altem Ascites finden sich anscheinend cytotoxische Inhibitoren, welche die Virulenz frischer Ascitescellen herabzusetzen vermögen.

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FURTHER OBSERVATIONS ON THE PATHOGENESIS OF RABIES IN GUINEA-PIGS AFTER EXPERIMENTAL INFECTION WITH THE FLURY STRAIN

by

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In previous papers (HUYGELEN and MORTELMANS 1958, 1959) experiments were reported on the pathogenesis of rabies in guinea-pigs following experimental inoculation with the Flury strain of rabies virus into the muscles of the hind leg. The virus content of the different portions of the central nervous system was titrated during the incubation period, during the period of clinical symptoms and after death. The results demonstrated that the virus first multiplied in the lumbosacral cord and subsequently spread in the direction of the brain. A close parallelism was observed between these virus titers and the course of the clinical symptoms, characterized by ascending paralysis.

In the experiments here reported an investigation has been made into the dissemination of rabies virus after intramuscular inoculation into the foreleg and after injection by the intravenous route.

MATERIALS AND METHODS.

All experiments were carried out on guinea-pigs, weighing between 300 and 400 g, and one month old albino mice, obtained from the breeding colony of the laboratory.

The Flury strain of rabies virus, originally obtained from Dr. KOPROWSKI, was at its 48th egg passage level and it titrated $10^{-3,85}$ in mice. For both intramuscular and intravenous inoculations we used 1 ml of a 40 % chick embryo suspension, but the material used

for the intravenous injections had to be clarified previously by centrifugation at 1500 revolutions per minute, because the large particles tended to obstruct the small gauge needles. The intravenous injection was given into the dorsal veins of the penis.

Suspensions of the different portions of the central nervous system of the guinea-pigs were made in normal saline after centrifugation at 1500 revolutions per minute for ten minutes. Four to six mice were used for each dilution and all mice were observed for 21 days.

Experiments.

In a first experiment 25 guinea-pigs received one ml of a 40 % chick embryo suspension intramuscularly into the right foreleg. At regular intervals after inoculation one or two guinea-pigs were killed and samples taken from their brain hemispheres, bulb, cervico-thoracic cord and lumbosacral cord. Suspensions of each of these samples were injected intracranially into mice.

On the whole 14 guinea-pigs were killed and examined for the presence of virus in the different segments of the central nervous system; 11 animals remained for control: 10 died between postinoculation days 9 and 12 and one survived.

The first symptoms in the guinea-pigs left as control animals were observed on the 7th day and were characterized by paralysis of the right foreleg, followed by paralysis of the left foreleg and subsequently by involvement of the bulbar centers and death. Generally the course of the disease was much shorter than in guinea-pigs inoculated into the hind legs.

All guinea-pigs killed during the first six postinoculation days were apparently perfectly normal. One animal (A) killed on the 7th day showed paralysis of the right foreleg and the other (B) paralysis of both forelegs and symptoms of bulbar paralysis. The guinea-pig sacrified on the 8th day was nearly completely paralyzed.

The results, summarized in table 1, show that no virus could be recovered from the guinea-pigs killed during the first three days. In one of the guinea-pigs killed on the 4th day and in one of those killed on the 5th day virus could be detected in the cervico-thoracic cord. In the animal sacrificed on the 6th day, the virus was present in the cervico-thoracic cord and also, but in smaller quantities in the bulb and in the lumbosacral cord; no virus could be isolated from

TABLE 1.

Mortality rate of mice inoculated intracerebrally with suspensions of different portions of the central nervous system of guinea-pigs, killed at stated intervals following intramuscular inoculation into the foreleg.

Dilution	Guinea-pigs killed . . . . day after inoculation													
	1		2		3		4		5		6	7		8
	A	B	A	B	A	B	A	B	A	B		A	B	
Brain 1/5	0+/5°	0/5	0/6	0/5	0/6	0/4	0/3	0/3	—	—	—	—	—	—
10^{-1}	0/5	—	—	—	—	—	—	—	0/4	0/3	0/4	3/3	1/4	4/4
10^{-2}	—	—	—	—	—	0/3	0/4	0/4	0/4	0/4	0/3	4/4	1/4	4/4
10^{-3}	—	—	—	—	—	—	—	—	0/4	0/4	0/3	4/4	0/3	2/4
10^{-4}	—	—	—	—	—	—	—	—	0/4	0/4	0/4	1/4	0/4	2/4
10^{-5}	—	—	—	—	—	—	—	—	—	—	—	0/4	0/4	0/4
10^{-6}	—	—	—	—	—	—	—	—	—	—	—	—	—	0/4
Bulb 1/5	0/5	0/6	0/5	0/4	0/5	0/3	0/3	0/3	—	—	—	—	—	—
10^{-1}	0/4	—	—	—	—	—	—	—	0/3	0/3	2/2	—	—	4/4
10^{-2}	—	—	—	—	—	0/3	0/4	0/4	0/4	0/4	3/4	—	—	4/4
10^{-3}	—	—	—	—	—	—	—	—	0/4	0/4	1/4	—	—	4/4
10^{-4}	—	—	—	—	—	—	—	—	0/4	0/4	0/4	—	—	2/2
10^{-5}	—	—	—	—	—	—	—	—	—	—	—	—	—	3/4
10^{-6}	—	—	—	—	—	—	—	—	—	—	—	—	—	0/4
Cervico thoracic cord 1/5	0/5	0/5	0/4	0/5	0/3	0/2	0/3	3/3	—	—	—	—	—	—
10^{-1}	—	—	—	—	—	—	—	—	0/3	1/4	3/3	3/3	4/4	4/4
10^{-2}	—	—	—	—	—	0/3	0/3	1/3	0/4	0/4	4/4	2/2	4/4	4/4
10^{-3}	—	—	—	—	—	—	—	—	0/4	2/3	3/3	3/3	4/4	3/3
10^{-4}	—	—	—	—	—	—	—	—	0/3	0/4	2/3	3/4	0/3	4/4
10^{-5}	—	—	—	—	—	—	—	—	—	—	—	1/4	0/4	1/4
10^{-6}	—	—	—	—	—	—	—	—	—	—	—	—	—	1/4
Lumbosacral cord 1/5	0/5	0/5	0/6	0/5	0/4	0/3	0/4/	0/3	—	—	—	—	—	—
10^{-1}	—	—	—	—	—	—	—	—	0/3	0/4	1/4	4/4	3/4	4/4
10^{-2}	—	—	—	—	—	0/4	0/3	0/4	0/4	0/3	1/4	0/3	2/4	4/4
10^{-3}	—	—	—	—	—	—	—	—	0/4	0/4	0/4	0/4	1/3	4/4
10^{-4}	—	—	—	—	—	—	—	—	0/4	0/4	0/4	0/2	0/4	2/4
10^{-5}	—	—	—	—	—	—	—	—	—	—	—	0/4	0/4	0/4
10^{-6}	—	—	—	—	—	—	—	—	—	—	—	—	—	0/4

+ = number of mice which died from rabies.

° = number of mice inoculated, not included those which died from an intercurrent disease or from cerebral trauma.

the brain hemispheres. From the 7th day onwards the virus was present in all portions of the central nervous system, but in both animals killed on that day, the titer was higher in the cervico-thoracic cord. In the guinea-pig killed on the 8th day the virus titer of the cervico-thoracic cord and the bulb were nearly identical, whereas the titers of the brain and the lumbosacral cord were lower.

In a second experiment we tested the susceptibility of guinea-pigs to intravenous inoculation of the Flury strain. In a preliminary test eight animals were injected into the veins of the penis with 1 ml of a centrifuged 40% chick embryo suspension; two guinea-pigs received 0.1 ml of the same suspension subcutaneously in the penis in order to determine if a small amount of virus, introduced accidentally into the surrounding tissues outside the vein, would be able to induce infection. Both animals were observed for 50 days and showed no signs of illness. Five other guinea-pigs received 1 ml of the same suspension intramuscularly into the hind leg. Three of them died after having shown a typical ascending paralysis.

Seven guinea-pigs which had received the virus intravenously, died between the 8th and 11th day after infection and one survived. They showed a rapidly fatal bulbar paralysis and one of them showed rhythmic contractions of the diaphragma for several hours before death.

In a further experiment 16 guinea-pigs were inoculated intravenously by the same method. On the 3rd day four guinea-pigs were killed, samples were taken from the brain hemispheres, the bulb, the thoracic cord and the lumbo-sacral cord of each animal and suspensions ($^1/_5$ in normal saline) of each portion were inoculated intracerebrally into mice. Four guinea-pigs were sacrificed on the 4th day, three on the 5th day and two on the 6th day. Three animals were left for control: two of them died on the 8th day with symptoms of bulbar paralysis and one on the 9th day with symptoms of ascending paralysis.

The results, summarized in table 2, demonstrate that no virus could be isolated during the first five days. In the two animals, killed on the 6th day, the virus was present in each portion of the central nervous system. The concentration however seemed to be much higher in the anterior part (brain hemispheres and bulb). No titration was carried out, but both the mortality rate and the length of the incubation period in the inoculated mice indicate that the virus content was higher in the brain and the bulb. In one of

TABLE 2.

Mortality rate of mice inoculated intracerebrally with suspensions of different portions of the central nervous system of guinea-pigs, killed at stated intervals after intravenous injection.

Dilution		Guinea-pigs killed . . . . days after inoculation												
		3				4				5			6	
		A	B	C	D	A	B	C	D	A	B	C	A	B
Brain	1/5	$0^{+}/4^{\circ}$	0/4	0/4	0/4	0/2	0/5	0/5	0/5	0/5	0/5	0/5	6/6	4/4
Bulb	1/5	0/4	0/3	0/4	0/3	0/4	0/4	0/5	0/5	0/5	0/4	0/4	6/6	5/5
Thoracic cord	1/5	0/4	0/4	0/3	0/4	0/5	0/5	0/5	0/2	0/4	0/5	0/5	1/5	5/5
Lumbo-sacral cord	1/5	0/4	0/4	0/4	0/4	0/2	0/5	0/5	0/5	0/3	0/5	0/4	3/6	2/4

$^{+}$ = number of mice which died from rabies.

$^{\circ}$ = number of mice inoculated, not included those which died from an intercurrent disease or from cerebral trauma.

the animals suspensions made from the brain hemispheres and the bulb, killed all mice between the 6th and 8th day. A suspension from the thoracic cord only killed one mouse on the 12th day and a suspension from the lumbosacral cord killed two mice on the 11th and 12th day. In the other animal similar results were obtained with suspensions of the brain hemispheres and the bulb. The thoracic cord, though comparatively more virulent than that of the other guinea-pig, contained less virus than the anterior parts of the nervous system, since the mice died after a longer incubation period (11 days). The lumbosacral cord contained only a small amount of virus: only half of the mice died (mean survival time: 11 days).

Discussion.

The above reported results on the spread of rabies virus after intramuscular inoculation confirm those previously recorded (Huygelen and Mortelmans 1958, 1959). The first segment of the central nervous system, invaded by the virus, corresponded with the site of inoculation. The first symptom observed in guinea-pigs, inoculated into the foreleg, consisted of paralysis of the inoculated limb, followed by paralysis of the opposite limb. On the other hand the virus could be recovered from the cervico-thoracic cord two

days before it could be isolated from other portions of the cord or the brain. From the 6th day on, *i.e.*, after local multiplication in the cervico-thoracic cord, it spread rapidly in both directions to the anterior and posterior parts of the central nervous system. The same striking parallelism as previously reported, between the virus distribution and the clinical symptoms, could be noticed. The slow ascending paralysis, observed after injection into the hind leg, was absent after inoculation into the foreleg and the course of the disease was shorter because of the more rapid involvement of the vital centers of the bulb.

Recently KRAUSE (1957) emphasized the rôle of the blood in the dissemination of rabies virus. In several instances he recovered virus from the blood some time after inoculation. Following an intramuscular injection a large amount of virus is undoubtedly resorbed by the blood and disseminated throughout the body, but does this fact involve that it is this part of the virus, taken up by the blood, which is actually responsible for the establishment of the infection in the nervous tissues? In our experiments as much as 1 ml of virus suspension was injected in most instances and the fairly quick resorption of the local swelling indicated that a great deal of virus must have been taken up by the circulating blood. Nevertheless the first symptoms and the first virus multiplication in the central nervous system always occurred near the site of inoculation.

In our second experiment the virus was inoculated by the intravenous route. It regularly induced infection by this way and apparently was at least as infective by the intravenous as by the intramuscular route. The paralysis began in all cases, except one, in the anterior part of the central nervous system. These observations are in accordance with those of DI VESTEA and ZAGARI (1889), who noticed that in many instances the virus shows some predilection for the bulb when inoculated intravenously, whereas PASTEUR *et al.* (1884) concluded from their experiments in dogs, that following intravenous inoculation the primary virus multiplication occurred in the lumbosacral cord.

On the other hand our experiments confirm the work of PANISSET and DISCHAMPS (1920) who, working with fixed rabies virus, proved the high susceptibility of guinea-pigs to intracardial inoculation of the virus.

The results of the above reported experiments prove that both

blood and nerves may play a rôle in the dissemination of rabies virus. The actual pathway of street virus after natural infection remains unknown. Further work is required to demonstrate whether the virus normally travels by way of the blood stream or by the neural route, or, whether it may use a combination of both, as it has been observed in experimental poliomyelitis virus infections (Bodian, 1954).

Summary.

Following inoculation of guinea-pigs with the Flury strain of rabies virus in the muscles of the foreleg, the virus could be recovered from the cervico-thoracic cord on the 4th day. From the 6th day on the virus spread in both directions, to the brain and to the lumbosacral cord. There was a close parallelism between the virus distribution and the clinical symptoms: the first signs of paralysis were observed in the inoculated leg.

Intravenous inoculation into the veins of the penis resulted in a mortality rate at least as high as that induced by intramuscular injection. The symptoms and the virus distribution demonstrated that the virus first invaded the anterior part of the central nervous system.

From these experiments and others previously reported, we may conclude that after intramuscular inoculation the first segment of the central nervous system, invaded by the virus, corresponds with the site of inoculation, whereas after intravenous inoculation no such correlation exists.

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A METHOD FOR THE MEASUREMENT OF ANTIBODIES AGAINST *HEMOPHILUS PERTUSSIS* IN SERA

by

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(Received October 15, 1959).

INTRODUCTION.

The antibody content of *Hemophilus pertussis* sera is usually determined with the technique of the tube-agglutination, wherein the agglutinogen consists of a fresh *H. pertussis* phase I bacteria suspension, prepared from a culture on the well known Bordet-Gengou agar. In our laboratory we frequently needed such a fresh suspension in order to determine agglutination titers from sera. As the preparation of such fresh suspensions is taking up much time, we decided to search for a stable *H. pertussis* antigen, with the same properties as a fresh bacterial suspension. To obtain a further saving of time, a modification of the usual technique of tube-agglutination was desirable. We adapted the method with porcelain tiles known from the field of virology for our purpose.

In the present paper the preparation of coloured *H. pertussis* antigen and a new agglutination technique are described and their usefulness is demonstrated.

MATERIALS AND METHODS.

S t r a i n. The *H. pertussis* strain used, was one of our laboratory strains (VAN DER VEEN, 1951). Cultivation took place on the Bordet-Gengou (B.G.) medium.

A n t i s e r u m. The serum used was a pool of sera from rabbits, immunised by repeated intravenous injection with a suspension of

H. pertussis phase I bacteria cultivated on B.G. agar and killed with merthiolate 1 : 10,000. The average agglutination titer against a fresh bacterial suspension was 1 : 15,000 (final dilution of the serum).

Preparation of the antigen. Dessicated bacteria taken from a culture dried and sealed under vacuum, were suspended in saline and inoculated on a B.G. plate. After 48 hours incubation at 37°C., the growth was transferred to 2 B.G. plates. After incubation again for 48 hours, the collected bacteria were inoculated on 10 B.G. plates. The growth on these was used as seed for 100 B.G. plates. From these 100 plates the bacteria were scraped off with a glass spatula and collected in saline. The suspension was centrifuged at 3000 r.p.m. for one hour. Hereafter the supernatant was discarded, the cells were washed in saline and centrifuged again. The packed cells were suspended in saline, shaken with glass beads until a homogeneous suspension was obtained and filtered through a plug of cotton wool to remove small clumps of agar. The filtered suspension had to contain about 10^{12} bacteria per ml. After thoroughly mixing with two volumes of ethanol the cells were precipitated and stored for three days at 4°C. The supernatant was now pipetted off for the larger part and the rest removed after centrifugation during half an hour. The compact precipitate was resuspended in the original volume of saline with 0,5 % formalin, and treated with ultrasonic waves (22 kilocycles, 1 kilowatt during 5 min.) [1]) in order to make a completely homogeneous suspension. To 0.25 ml of the suspension were added 0.5 ml hematoxylin solution (760 mg hematoxylin in 50 ml ethanol) and the mixture was shaken for 4–6 hours at 37°C. Now saline was added till 10 ml was obtained, and the mixture was centrifuged at 3000 r.p.m. for 30 min. The precipitate was washed three times with saline and centrifuged again. Finally saline was added till 4 ml was obtained in order to get a blue-purple antigen with about 5×10^{10} cells per ml.

Agglutination test. With the stained antigen described above it was possible to measure the agglutinin content of sera in the same manner as the hemagglutination of viruses described by SALK (1944) and modified by VAN DER VEEN (1950). With a Pasteur pipette a series of twofold dilutions was made in the cups of a

[1]) We are indebted to the Natuurkundig Laboratorium N.V. Philips' Gloeilampenfabrieken, Eindhoven, for co-operation on this point.

TABLE 1.

Agglutination patterns of two different antigens with a *H. pertussis* serum (tube agglutination).

Final dilution of serum	300	600	1200	2400	4800	9600	19200	38400	76800	153600
Coloured antigen 5×10^9 bacteria/ml	++++	++++	++++	++++	++++	++++	+++	++	+	–
Fresh suspension 5×10^9 bacteria/ml	++++	++++	++++	++++	++++	++++	++	+	±	–

TABLE 2.

Agglutinin absorption test from a *H. pertussis* vaccin supernatant.

	Adsorbent	Conc. of adsorbent	final dilution of serum added for agglutinin absorption							
			300	600	1200	2400	4800	9600	19200	38400
A	Vaccine supernatant	1/1	++++	++++	++++	+++	+	–	–	–
		1/2	++++	++++	++++	++++	+++	–	–	–
		1/4	++++	++++	++++	++++	++++	+++	+	–
	None (serum 1 : 150)	–	++++	++++	++++	++++	++++	++++	+	–
B	Vaccine supernatant	1/1	++++	++++	++++	+++	++	–	–	–
		1/2	++++	++++	++++	++++	++++	++	–	–
		1/4	++++	++++	++++	++++	++++	++	+	–
	None (serum 1 : 150)	–	++++	++++	++++	++++	++++	+++	+	–

A: titration with coloured antigen in porcelain tiles (5×10^9 bacteria/ml).
B: titration in tubes with fresh bacterial suspension (5×10^9 bacteria/ml).

porcelain tile. In each cup are two drops of saline. To the first cup are added two drops of serum and, after mixing, two drops of this mixture are placed in cup 2 and so on. Then to each cup are added two drops of antigen. After thoroughly mixing the respective contents of each cup with a clean glass rod the tiles are placed in Petri dishes, to prevent evaporation, and incubated for two hours at

37°C. After standing during 18 hours at room temperature the patterns are read. Partial agglutination is expressed as 1, 2, and 3 plus, total as 4 plus agglutination, depending on the size of the patterns.

Results.

The antigen and the agglutination method described above were used in many agglutination tests and also in the agglutinin absorption test (see for details about the agglutinin absorption test *e.g.* Flosdorf *et al.*, 1939,1940). In several experiments it was shown that the agglutination titers against sera were the same as those with fresh bacteria suspensions. Examples are given in tables 1 and 2.

The antigen was easy to prepare and very stable. After storage for 8 months in the refrigerator, there was no trace of autolysis and the titer against standard serum was unchanged.

We tried also to make homogeneous suspensions without the use of ultrasonic waves. However the results with shaking, fast stirring or mixing with a vibration mixer were less satisfactory because many clumps of bacteria were not disrupted. Such antigens were less stable and did not give good agglutination patterns.

Only resuspension of the compact precipitate, obtained after precipitation with ethanol and centrifugation, in saline with formalin yielded a usable antigen (de Bock and Worst-van Dam, 1960).

Summary.

The preparation of a stable coloured antigen of *Hemophilus pertussis* and a new agglutination method are described.

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LACTOBACILLUS BULGARICUS (LUERSSEN ET KÜHN) HOLLAND

by

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1. Introduction.

The definition of *Lactobacillus bulgaricus* as given in Bergey's Manual (1957) in our opinion includes three species of bacteria that have been described by Orla-Jensen (1943): *Thermobacterium bulgaricum, Tbm helveticum* and *Tbm jugurt*. As we had previously found intermediate forms between *Tbm helveticum* and *Tbm jugurt*, in an earlier publication (1956a) we proposed uniting these two species under the name of *Tbm helveticum* or *Lactobacillus helveticus*.

A great part of the existing confusion about the taxonomy of the aforementioned micro-organisms is no doubt a consequence of the incompleteness of the description given by Luerssen and Kühn (1908) of their *Bacillus bulgaricus* isolated from yogurt. The fact that this bacterium probably formed laevorotatory lactic acid indicates that *Bacillus bulgaricus* must be identical with *Tbm bulgaricum* as described by Orla-Jensen and not with *Tbm helveticum*.

According to Orla-Jensen (1913) all lactobacilli isolated from real yogurt always formed laevorotatory lactic acid. This author (1943) also found that these bacteria only fermented a limited number of substrates, *e.g.* never maltose or trehalose, and only formed about 1.7% acid in milk. *Tbm helveticum*, on the other hand, was found to produce about 2.7 % inactive lactic acid in milk and often fermented maltose or trehalose or both (Orla-Jensen, 1943; de Man, 1956a).

In Bergey's Manual the assumption has been made that the lactobacilli described by Grigoroff (1905), Cohendy (1906), Bertrand and Duchacek (1909) and White and Avery (1910) were identical with the *Bacillus bulgaricus* of Luerssen and Kühn,

although the bacteria described by the first mentioned authors have certain characteristics of *Tbm helveticum*.

De Vleeschauwer *et al.* (1954) and Kundrat (1958) isolated a number of strains of lactobacilli from yogurt, de Vleeschauwer presumably from yogurt made in Belgium and Kundrat from samples of yogurt originating from Yugoslavia. Nearly all the strains isolated by de Vleeschauwer and all the strains of Kundrat had the same fermentation characteristics as the strain of *Thermobacterium bulgaricum* described by Orla-Jensen (1943). Neither of these authors determined the configuration of the lactic acid formed by the bacteria. The primary intention of the experiments reported in this paper was to find out whether the lactobacilli isolated by us from yogurt did also form laevorotatory lactic acid.

2. Cultures and methods.

a. Cultures.

The eight cultures investigated were all present in the N.I.Z.O. collection and had been previously isolated from samples of yogurt made in eight different factories in the Netherlands.

b. Methods.

The methods used have been described in an earlier publication (de Man, 1956b). The bacteria were incubated at 37° C.

As a basal medium for the study of salt and taurocholate resistance we used medium GM containing the following ingredients:
10 g Oxoid peptone, 10 g meat extract (Lab Lemco, Oxo), 5 g Difco yeast extract, 20 g glucose, 1 ml Tween 80, 2 g K_2HPO_4, 0.5 g sodium acetate $5H_2O$, 0.2 g monopotassium citrate, 0.14 g $MgSO_4$ $7H_2O$, 1 litre distilled water; pH 6.5.

This medium is virtually identical to the growth medium for lactobacilli which has been developed by de Man, Rogosa and Sharpe (to be published).

3. Results.

The results obtained have been recorded in Table 1. In all cases the lactic acid isolated was laevorotatory. Of the sugars investigated only glucose was fermented. All these properties are exactly as described by Orla-Jensen.

These results indicate that the description of *Lactobacillus bulgaricus* in Bergey's Manual should indeed be altered so as to

TABLE 1.

Examination of strains of *Lactobacillus* isolated from yogurt.

Strain *)	pH after 48 h growth in			in milk		pH after growth in medium with								
	GM	GM + 2% NaCl	GM + 4% NaCl	% lactic acid	$[\alpha]_D^{20}$ isolated zinc lactate	cello-biose	glucose	mal-tose	manni-tol	meli-biose	rham-nose	sucrose	salicin	treha-lose
blank	5.95	6.10	5.90			6.75	6.65	7.10	7.40	6.70	6.60	7.30	7.30	7.30
3501	4.10	4.00	5.65	1.71	8.0	6.50	4.10	6.80	7.30	6.60	6.40	7.15	7.10	7.05
3502	4.00	4.65	5.80	1.94	6.5	6.35	4.10	6.80	7.25	6.50	6.45	7.20	7.20	7.20
3503	3.70	4.30	5.80	1.87	7.8	6.60	3.90	6.95	7.30	6.40	6.40	7.00	7.20	7.10
3504	3.80	3.75	5.50	2.22	7.7	6.20	4.15	6.70	7.25	6.40	6.40	6.95	7.00	7.15
3505	3.65	4.00	5.50	1.89	6.7	6.50	4.10	6.80	7.20	6.50	6.45	7.10	7.10	7.20
3506	4.80	6.10	5.70	1.56	6.7	6.45	4.15	6.85	7.30	6.60	6.50	7.10	7.20	7.20
3507	3.70	3.80	5.55	1.63	6.7	6.40	4.00	6.75	7.20	6.60	6.35	7.05	7.20	7.10
3508	3.65	4.10	5.65	1.78	8.0	6.35	3.95	6.80	7.30	6.50	6.40	7.00	7.15	7.00

*) All strains had the following characteristics:

Gram	+	Gas from glucose	—
Catalase	—	Growth at 45°	+
Motility	—	Growth at 15°	—
Nitrate reduction	—	Growth with 2% taurocholate	—
		Volatile acid production	< 0.07%

exclude *L. helveticus*. This has subsequently been confirmed by ROGOSA and SHARPE (1960) by means of other criteria for classification.

The amount of acid formed in milk never reached the amount which is characteristic for *Lactobacillus helveticus*, *viz*. 2.7%.

The strains did not grow in a medium with 2% sodium taurocholate, in accordance with the findings of WHEATER (1955) and KUNDRAT (1958). With one exception all our strains tolerated 2% sodium chloride in the medium, in accordance with the findings of KUNDRAT. According to WHEATER, *L. bulgaricus* could not grow with 2% sodium chloride, neither did the strains isolated by DE VLEESCHAUWER. Secondary circumstances must have caused these differences in results.

Summary.

From the results of the experiments described, as well as from the literature, it becomes clear that the definition of *Lactobacillus bulgaricus* given in Bergey's Manual should be altered. This bacterium produces laevorotatory (D) lactic acid almost exclusively and ferments a limited number of sugars only, *e.g.* not maltose, saccharose or trehalose.

All strains grew in a medium with 2% sodium chloride but no appreciable growth occurred with 4%, nor with 2% sodium taurocholate.

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UEBER DIE BEEINFLUSSUNG DER VIRULENZ VON TUMOREN DURCH CHEMOTHERAPEUTICA

EINFLUSS VON BAYER E 39 AUF DIE VIRULENZ DES KREBS-2-ASCITESCARCINOM

von

W. A. COLLIER und **M. DE WIT**

(Empfangen 25. August 1959).

Nachdem Collier und de Wit (1960) darauf hingewiesen hatten, dass bei Ascitestumoren die Virulenz graphisch dargestellt werden kann, und dass dergleichen Virulenzcurven ein deutliches Bild von der Beeinflussung der verschiedenen Factoren der Tumorcelle und des Wirtes geben, war es von Interesse, diese Methode auch auf Versuche über Chemotherapie bei Ascitestumoren anzuwenden.

Es ist bekannt, dass nur Versuche mit einer grösseren Anzahl der verschiedenartigsten Tumoren einen Eindruck von einer möglichen Eignung eines Therapeuticums für die menschliche Therapie zu geben vermögen. Es scheint aber die Neigung zu bestehen, bei dergleichen Versuchen immer mehr die in Ascitesform wachsenden Tumoren heranzuziehen, da sich mit ihnen so ausserordentlich einfach arbeiten lässt.

Auf die Eignung der Ascitestumoren für chemotherapeutische Untersuchungen wiesen u.a. Sugiura und Creech (1956) hin. Genau wie Lettré (1950) wenden sie Gewichtsmessung an, gelegentlich auch Messung der Ascitesmenge zehn Tage nach der ersten Applikation der untersuchten Substanz. Sie behandeln sieben Tage lang.

Für die Beurteilung chemotherapeutischer Präparate benutzen Furst, Cutting und Gross (1955) die Ueberlebenszeit bei mit 10–15 $\times$ 10^6 Ascitescellen intraperitoneal inficierten und intraperitoneal behandelten Tieren.

TABELLE 1.

Absterbezeiten (cumulatieve Sterblichkeit) im Therapieversuch mit E 39.
(E 39 $^1/_{10\,000}$ 0,5 10 ×, beginnend 1 Stunde nach Infection intraperitoneal, also ,,intratumoral'').

Infectionsdosis 0,2 ml ip = Cellenanzahl		Therapieversuche							Controllversuche							Dif-ferenz
		$^1/_1$	$^1/_{10}$	$^1/_{100}$	$^1/_{1000}$	$^1/_{10000}$	$^1/_{100000}$	log $\frac{1}{LD_{50}}$	$^1/_1$	$^1/_{10}$	$^1/_{100}$	$^1/_{1000}$	$^1/_{10000}$	$^1/_{100000}$	log $\frac{1}{LD_{50}}$	
		8 000 000	800 000	80 000	8 000	800	80		8 000 000	800 000	80 000	8 000	800	80		
Tag:	E 39 ip															
0.	0,5															
1.	0,5															
3.	0,5															
4.	0,5															
5.	0,5															
8.	0,5															
10.									$^2/_6$							
11.	0,5								$^3/_6$						07	0,7
12.		$^1/_6$							$^6/_6$						1,2	1,2
13.		,,							,,	$^1/_6$					1,3	1,3
14.	0,5	,,							,,	$^3/_6$					1,7	1,7
15.		,,							,,	$^4/_6$					2,0	2,0
17.	0,5	$^3/_6$						0,7	,,	,,	$^1/_6$				2,1	1,4

20.	0,5	,,							,,	,,	,,					
21.		,,							,,	,,	,,	1/6			2,6	1,5
22.		,,	2/6					1,3	,,	,,	,,	,,				1,3
23.		,,	,,						,,	,,	3/6	2/6	1/6		3,2	1,9
24.		,,	3/6					1,5	,,	,,	,,	,,	,,			1,7
25.		,,	,,						,,	,,	4/6	,,	2/6		3,4	1,9
26.		,,	,,						,,	,,	,,	,,	,,	1/5	3,6	2,1
27.		,,	,,	1/6				1,7	,,	,,	5/6	,,	,,	2/5	3,9	2,2
28.		,,	,,	2/6				2,0	,,	,,	,,	,,	,,	3/5	4,1	2,1
29.		,,	4/6	3/6				2,3	,,	,,	,,	,,	,,	,,		1,8
31.		,,	,,	4/6	1/6			2,7	,,	,,	,,	,,	,,	,,		1,4
33.		,,	,,	,,	,,	1/6		2,8	,,	,,	,,	3/6	,,	,,	4,3	1,5
34.		6/6	,,	,,	,,	,,		2,9	,,	,,	,,	,,	,,	,,		1,4
38.		,,	,,	,,	,,	,,			,,	,,	,,	,,	3/6	4/5	4,7	1,8
39.		,,	,,	,,	,,	,,			,,	,,	,,	4/6	,,	,,	4,9	2,0
46.		,,	,,	,,	,,	,,			,,	,,	6/6	,,	,,	,,	5,2	2,3
49.		,,	,,	6/6	,,	,,		3,2	,,	,,	,,	,,	,,	,,		2,0
52.		,,	,,	,,	,,	,,			,,	,,	,,	,,	4/6	,,	5,4	2,2
54.		,,	,,	,,	3/6	2/6		3,7	,,	,,	,,	,,	,,	,,		1,7
55.		,,	,,	,,	,,	,,			,,	,,	,,	5/6	,,	,,	5,8	2,1
58.		,,	,,	,,	,,	3/6		3,9	,,	,,	,,	,,	,,	,,		1,9
70.		6/6	4/6	6/6	3/6	3/6	0/6	3,9	6/6	6/6	6/6	5/6	4/6	4/5	5,8	1,9

Die hier mitgeteilten Versuche wurden mit dem KREBS-2-Ascites-Carcinom durchgeführt. Als Chemotherapeuticum wurde das Cytostaticum ,,Bayer E 39'' gewählt, über das bereits eine ausgebreitete Literatur vorliegt. Von den diesbezüglichen Tierversuchen seien nur die von DOMAGK (1956, 1957) genannt, die erwarten liessen, dass ein Vergleich der Virulenzcurven von behandelten und nicht behandelten Versuchstieren ein deutliches Bild von der Wirkung des Präparates auf die Virulenz des verwendeten Ascitestumors ergeben würde. Die angewandte Technik ist bei Versuch 1 beschrieben.

DOMAGK erwartet von der Empirie die Beantwortung zweier Fragen: 1). Welche Stoffe haben einen sicheren cytostatischen Effect? 2). Welche morphologisch fassbaren Veränderungen rufen die Substanzen an Tumorcellen hervor? Wir möchten hier eine weitere Frage hinzufügen, die durch die Empirie zu beantworten ist: 3). Welchen Einfluss haben die Stoffe auf die Virulenz des Tumors?

Versuch 1.

Zunächst sei ein Versuch mitgeteilt, in dem eine Versuchsserie und eine Controllserie von je 6 × 6 Mäusen (Swiss-Stamm) mit je 0,2 ml einer Verdünnung von $^1/_1$, $^1/_{10}$, . . . bis $^1/_{100\,000}$ von 11 Tage alte Ascites intraperitoneal inficiert wurde. Zählung der Ascitescellen ergab, dass die Infection mit 8.000.000, 800.000 . . . bis 80 Cellen erfolgt war. Die Versuchsserie wurde 1 Stunde nach erfolgter Infection mit je 0,5 ml E 39 $^1/_{10\,000}$ intraperitoneal behandelt, was einer intratumoralen Therapie entsprechen würde. Eine weitere Behandlung mit gleicher Dosis erfolgte noch neunmal. Die Gesamtdosis betrug also 0,0005 g per Maus.

Der Verlauf des Versuches ist in Tabelle 1 zusammengestellt, aus der die Absterbezeiten der Mäuse hervorgehen. Angegeben ist die cumulative Sterblichkeit. Von der letzten Controllgruppe starb ein Tier intercurrent. Von beiden Serien wurde täglich die LD_{50} nach REED und MUNCH (1938) berechnet. Der Logarithmus des reziproken Wertes der täglichen LD_{50} ist gleichfalls in der Tabelle aufgenommen. Schliesslich ist noch die Differenz der Logarithmen zwischen Therapie- und Controll-Reihe berechnet.

Werden nun die Werte beider Reihen in Form von Virulenzcurven graphisch dargestellt, so erhält man in Figur 1 ein eindrucksvolles Bild von dem Einfluss des Cytostaticums E 39 auf die Virulenz des Ascitescarcinoms bei intraperitonealer Infection und intraperitonealer Behandlung. Die Differenz der LD_{50} zwischen Versuch

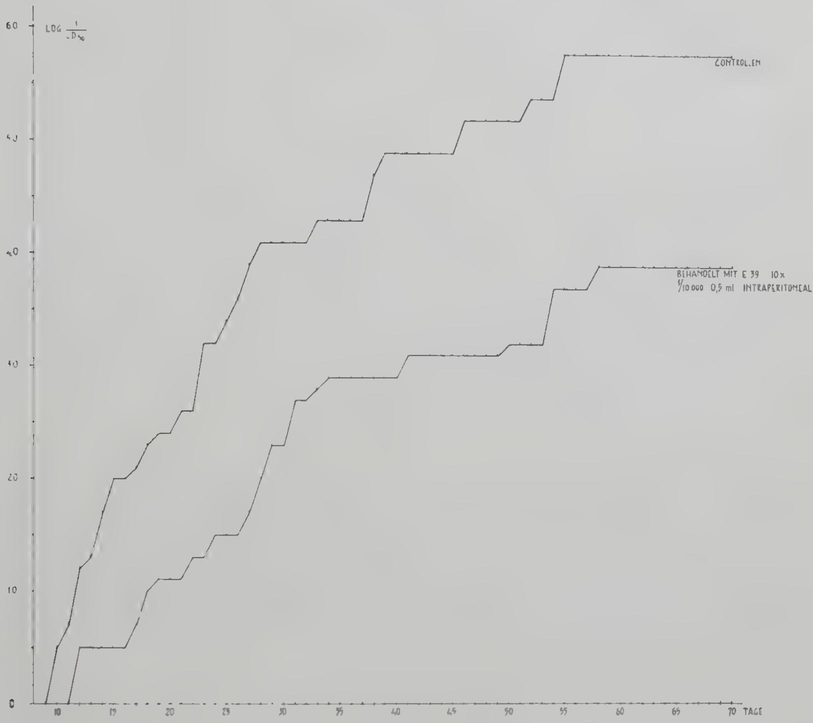

Fig. 1. Virulenzcurven von mit E 39 behandelten Mäusen und Controllmäusen (36 und 35 Tiere).

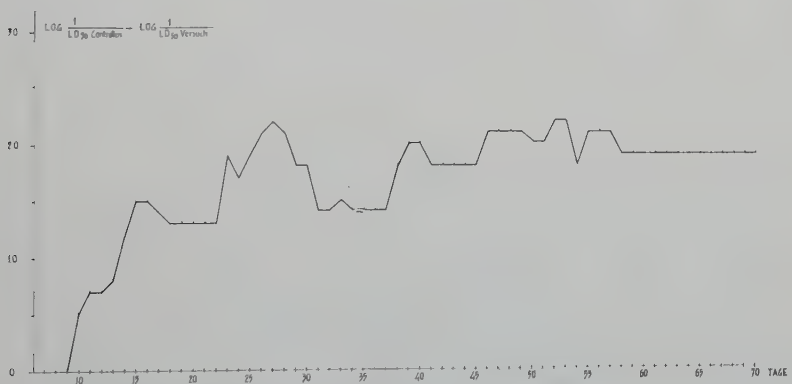

Fig. 2. Intervall zwischen der Virulenz von behandelten Mäusen und Controllmäusen, berechnet aus den Virulenzcurven.

und Controlle am Ende des Versuches ist = log 1,9, was einer Senkung der Virulenz um fast das 100-fache entspricht. Nicht so eindrucksvoll ist die Curve, die in Figur 2 den Verlauf der täglichen Differenzen erkennen lässt.

Vergleichsweise wurde noch in Figur 3 die durchschnittliche Lebensdauer in Tagen der behandelten Mäuse der Infectionsdosen von $^1/_1$ bis $^1/_{10\,000}$ mit denen der Controlltiere in Stabdiagrammen verglichen, wobei die am Leben bleibenden Mäuse als „100 Tage am Leben" gerechnet wurden. Weiterhin ist in Figur 4 die durchschnittliche Lebensdauer procentuell berechnet und dargestellt. Sowohl Figur 3 als auch Figur 4 ist wohl kaum so instructiv wie die in Figur 1 gegenübergestellten Virulenzcurven.

Versuch 2.

Drei Serien von je 6 × 6 Mäusen wurden mit je 0,2 ml von 11 Tage altem Ascites in den Concentrationen von $^1/_1$ bis $^1/_{100\,000}$ intraperitoneal inficiert. Die erste Serie wurde nach 24 Stunden mit 0,4 ml E 39 $^1/_{1000}$ intraperitoneal behandelt, die zweite Serie nach 2, 5, 8 und 12 Tagen mit je 0,2 ml E 39 $^1/_{1000}$. Die dritte Serie blieb als Controlle unbehandelt.

Aus Figur 5 ist zu ersehen, dass es zunächst zu einer gewissen Verminderung der Virulenz bei den einmal mit einer relativ hohen Dosis behandelten Mäusen kam. Zum Schluss aber stellte sich die normale Virulenz wieder her, und die Virulenzcurve der behandelten Mäuse überschritt etwas die der Controlltiere.

Deutlich war dagegen der Einfluss der 4-maligen Behandlung mit je 0,2 mg. Hier war die Virulenz wesentlich niedriger als die der Controllen, und am Ende des Versuches zeigte sich eine Differenz von log 1,3, was ungefähr $^1/_{20}$ der Virulenz der Controllen entspricht.

Versuch 3.

Sieben Serien von 6 × 6 Mäusen wurden mit je 0,2 ml 11 Tage altem Ascites in den Verdünnungen von $^1/_1$, $^1/_{10}$ . . . bis $^1/_{100\,000}$ intraperitoneal inficiert. Drei Serien wurden nach 1, 2, 3, 5, 6, 8, 10, 12, 14 und 16 Tagen mit kurzen Intervallen und drei Serien wurden nach 1, 3, 6, 8, 10, 13, 16, 19, 22 und 27 Tagen mit längeren Intervallen intraperitoneal mit je 0,5 ml E 39 in den Verdünnungen von $^1/_{10\,000}$, $^1/_{100\,000}$ bzw. $^1/_{1\,000\,000}$ behandelt. Dies entspricht einer „intratumoralen" Gesamtdosis von 0.0005, 0,00005 bzw. 0,000005 g per Maus. Die 7. Serie diente als Controllreihe.

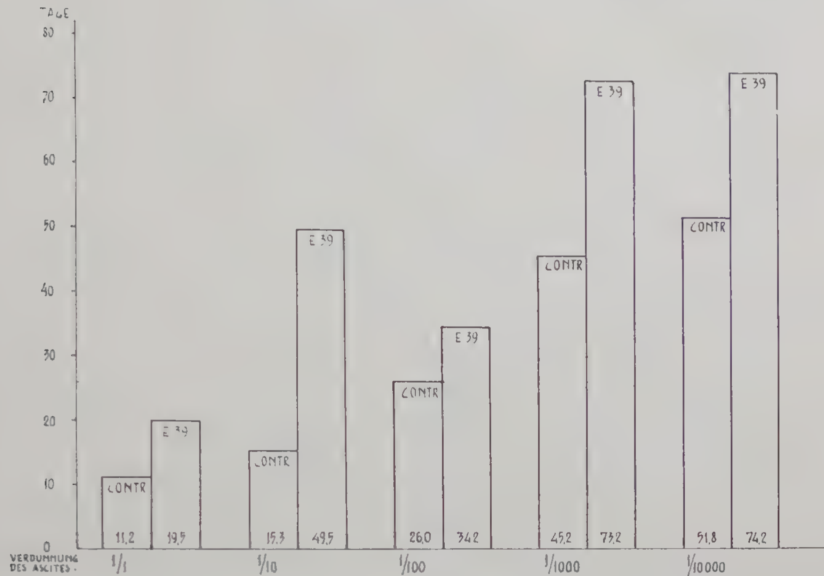

Fig. 3. Durchschnittliche Lebensdauer in Tagen von behandelten Mäusen und Controllmäusen (am Leben bleibende Tiere gerechnet als „100 Tage am Leben").

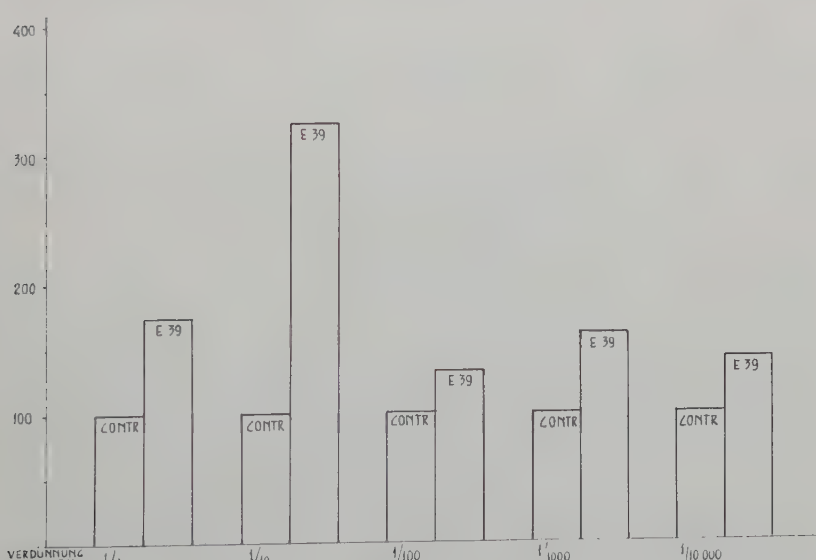

Fig. 4. Relative Lebensdauer behandelter Mäuse gegenüber den Controllmäusen = 100 (am Leben bleibende Tiere gerechnet als „100 Tage am Leben").

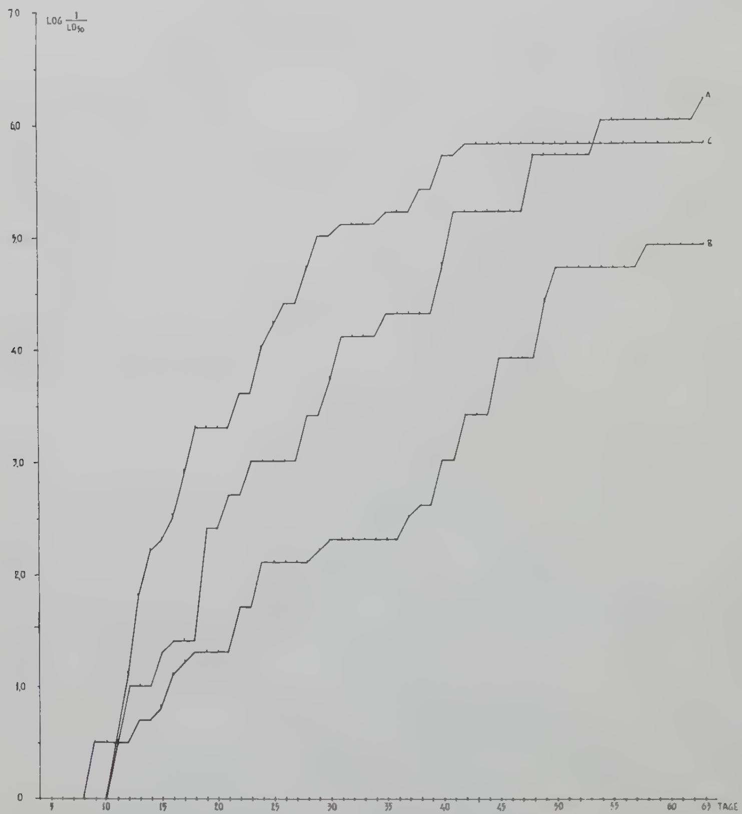

Fig. 5. Einfluss von kurzer Behandlung auf die Virulenz des Ascites-carcinoms. Virulenzcurven nach Behandlung mit
A = E 39 1 × $^1/_{1000}$ 0,4 ml
B = E 39 4 × $^1/_{1000}$ 0,2 ml
C = Controllmäuse.

In Figur 6 sind die Virulenzcurven der Versuche mit kurzem Behandlungsintervall wiedergegeben. Die Dosis von $^1/_{1\,000\,000}$ ist praktisch bedeutungslos, aber die Dosierung von $^1/_{100\,000}$ ergibt mit den Controllen eine Differenz von log 0,8, was einer Senkung der Virulenz um etwa das 7-fache entspricht. Bei der Dosierung von $^1/_{10\,000}$ ergibt sich mit den Controllen sogar eine Differenz von log 1,6, was einer Senkung der Virulenz um etwa das 40-fache entsprechen würde.

Etwas anders ist das Resultat, das sich bei den 3 Serien mit längeren Behandlungsintervallen zeigt. Die Virulenzcurven dieser

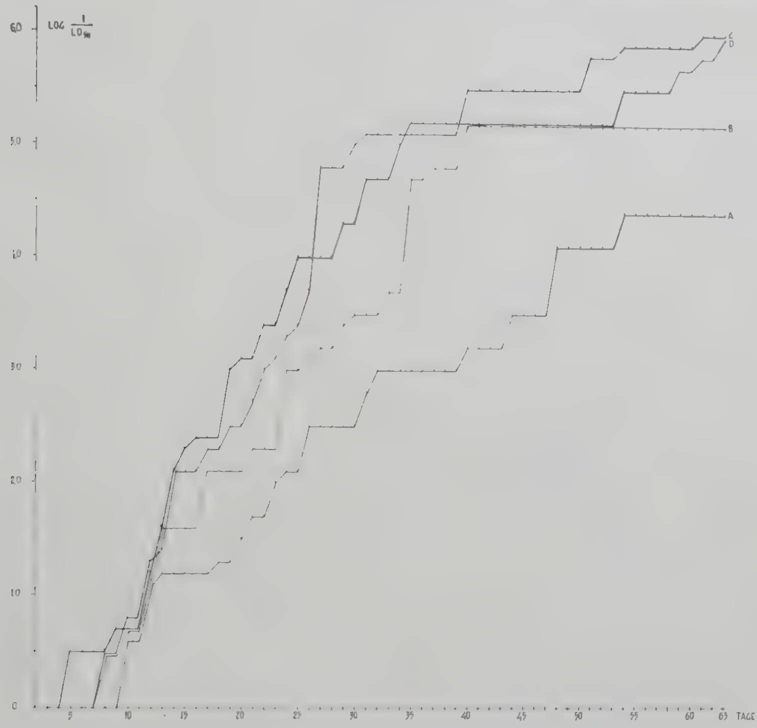

Fig. 6. Einfluss der intraperitonealen Behandlung mit E 39 auf die Virulenz nach einer Dosis von 10 ×
A = $^1/_{10\,000}$
B = $^1/_{100\,000}$
C = $^1/_{1\,000\,000}$
D = unbehandelte Controllmäuse.

Versuche sind in Figur 7 zusammengestellt. Es zeigte sich hier, dass alle drei Dosierungen ungefähr den gleichen Effekt hatten. Gegenüber den Controllen zeigten sich Differenzen von log 1,2 bzw. 1,3, was einer Virulenzsenkung um etwa das 12-13-fache entspricht.

Kann es bei der Behandlung mit längeren Intervallen auch nicht zu einer so starken Virulenzsenkung wie bei der Behandlung mit höheren Dosen bei kürzeren Intervallen, so schien doch bei den längeren Intervallen die Bedeutung der Höhe der Dosierung weniger gross zu sein.

Versuch 4.

In den früheren Versuchen von Collier und de Wit (1960) hatte sich gezeigt, dass die intracerebrale Infection mit Tumorascites

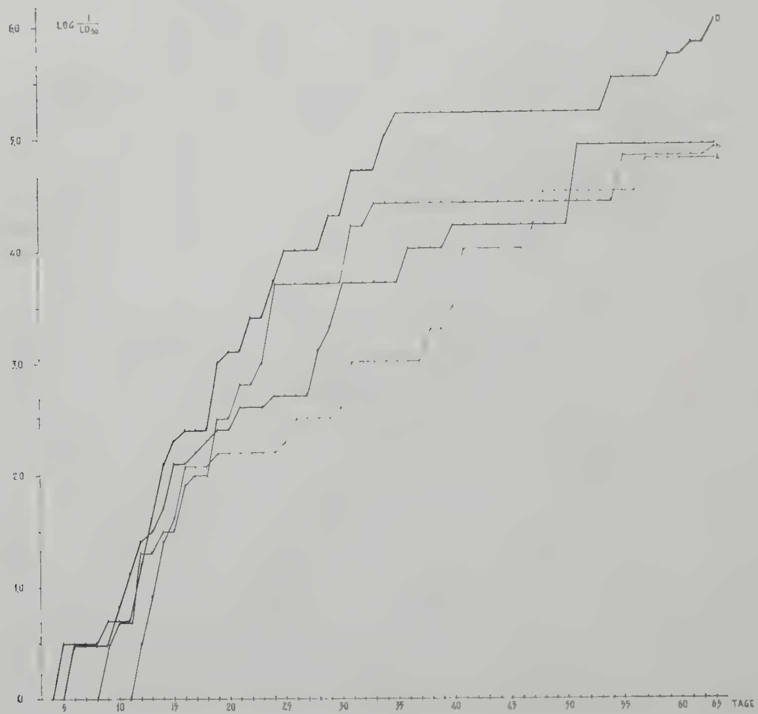

Fig. 7. Einfluss verschieden hoher Dosen von E 39 auf die Virulenz. Virulenzcurven nach Behandlung mit längeren Intervallen mit Dosen von 10 ×

A = $^1/_{10\,000}$
B = $^1/_{100\,000}$
C = $^1/_{1\,000\,000}$
D = Controllen

viel foudroyanter verläuft als die intraperitoneale oder subcutane.

Es wurden 2 Serien von 6 × 6 Mäusen intracerebral mit je 0,02 ml der Ascitesverdünnungen von $^1/_{10}$, $^1/_{100}$ . . . bis $^1/_{1\,000\,000}$ inficiert, und die eine Serie wurde nach 4 Stunden und nach 1, 2, 3, 4, 5, 7, 8, 9 und 12 Tagen mit je 0,5 ml E 39 in der Concentration von $^1/_{10\,000}$ subcutan behandelt, was also einer Gesamtdosis von 0,005 g per Maus entspricht.

In Figur 8 sind die beiden Virulenzcurven wiedergegeben. Es zeigt sich, dass bei der intracerebralen Infection keinerlei Einfluss zu erkennen war, was bei der Bösartigkeit des intracerebralen Verlaufes bereits erwartet werden konnte.

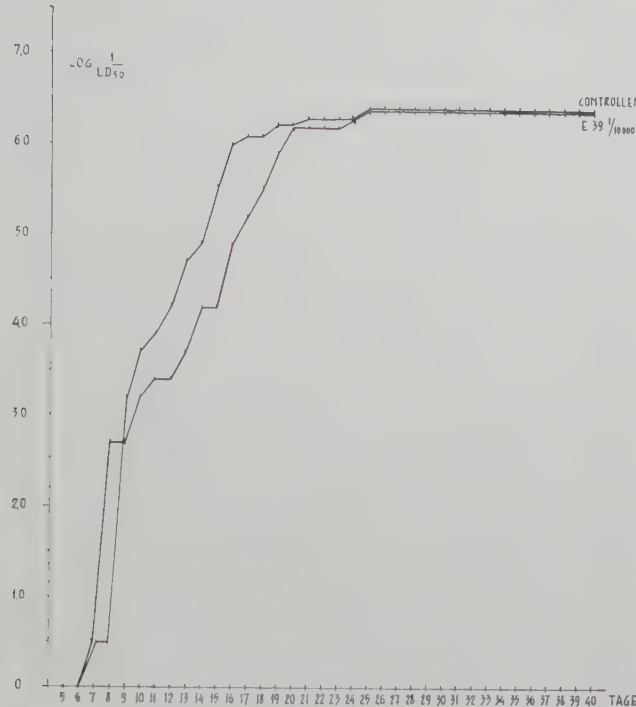

Fig. 8. Behandlung von intracerebraler Infection mit E 39 $^1/_{10\,000}$ 10 × 0,5 ml subcutan. Beginn der Therapie 4 Stunden nach Infection.

Versuch 5.

Es war von besonderem Interesse zu untersuchen, ob bei einer Maus mit starker Ascitesentwicklung die Virulenz durch „intratumorale" Behandlung beeinflusst werden konnte. Zu diesem Zweck wurde einer Maus mit 8 Tage altem Ascites etwas Material abgenommen und in den Verdünnungen von $^1/_{10}$ bis $^1/_{1\,000\,000}$ in einer Menge von je 0,02 ml intracerebral bei einer Serie von Mäusen austitriert. Die Ascitesmaus wurde mit 0,5 ml E 39 $^1/_{10\,000}$ intraperitoneal behandelt, was also einer intratumoralen Behandlung mit 0,00005 g entspricht. Nach 4 Stunden wurde wieder etwas Ascites abgenommen und in gleicher Weise intracerebral austitriert.

In Figur 9 sind die beiden Virulenzcurven wiedergegeben. Es zeigte sich deutlich, dass die Virulenz des Ascites 4 Stunden nach der lokalen Behandlung vermindert war. Am Ende der Beobachtungszeit zeigte sich zwischen den Versuchstieren und den

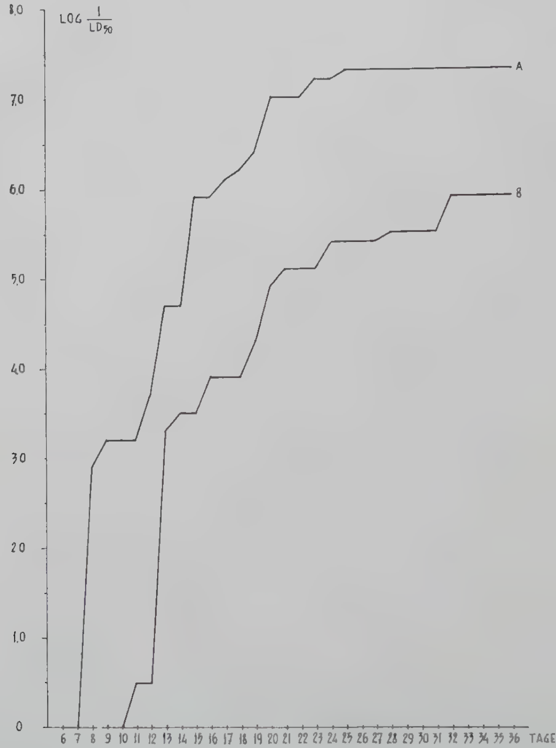

Fig. 9. Einfluss der intraperitonealen Behandlung mit E 39 auf die Virulenz des Ascites bei intracerebraler Verimpfung.

A = Virulenz vor Behandlung

B = Virulenz 4 Stunden nach intraperitonealer Behandlung mit E 39 $^1/_{10\,000}$ 0,5 ml.

Controllen eine Differenz der Virulenz von log 1,4, was einer Virulenzsenkung um das etwa 25-fache entspricht.

Versuch 6.

Es sei noch folgender Versuch ähnlicher Natur wiedergegeben. Eine Maus mit 14 Tage altem Ascites wurde zuerst punctiert und hierauf mit 1,0 ml E 39 $^1/_{1000}$ intraperitoneal behandelt, was einer sehr hohen Dosis von 0,001 g entspricht. Nach $1\frac{1}{2}$, 3 und $4\frac{1}{2}$ Stunden wurde wieder Ascites abgenommen. Alle 4 Ascitesproben wurden in der Menge von je 0,02 ml in den Verdünnungen von $^1/_{10}$ bis $^1/_{1\,000\,000}$ intracerebral austitriert.

Die Virulenzcurven sind in Figur 10 zusammengestellt. Ent-

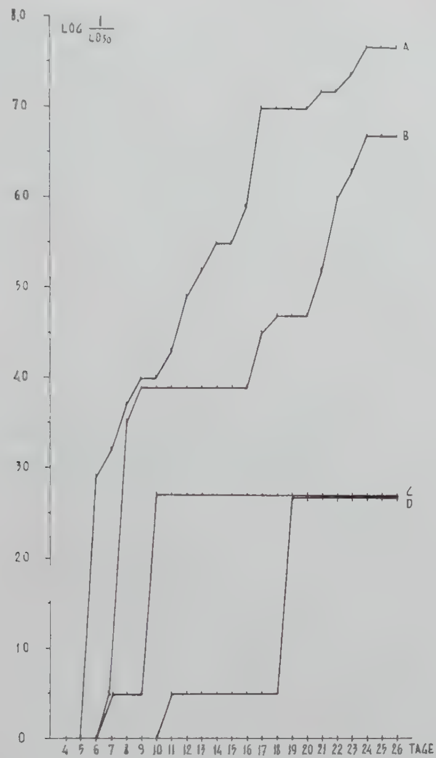

Fig. 10. Einfluss von E 39 1,0 ml $^1/_{1000}$ auf die Virulenz von Ascites bei intraperitonealer Verimpfung.
A = Virulenz vor der Therapie
B = Virulenz nach $1\frac{1}{2}$ Stunden
C = Virulenz nach 3 Stunden
D = Virulenz nach $4\frac{1}{2}$ Stunden.

sprechend der hohen Dosierung des Chemotherapeuticums ist auch der Einfluss auf die Virulenz sehr deutlich. Bereits nach $1\frac{1}{2}$ Stunden ist eine Senkung der Virulenz zu verzeichnen, die mit der Controllserie eine Differenz von log 1,0 ergibt. Viel stärker ist aber der Einfluss nach 3 und $4\frac{1}{2}$ Stunden. Hier betrug die Differenz mit der Controllgruppe log 5,0, was einer Senkung der Virulenz um das 100.000-fache entspricht.

Versuch 7.

Zum Schluss sei noch ein Versuch in vitro mitgeteilt. Es wurde 20 Tage alter Ascites von $^1/_{10}$ bis $^1/_{1\,000\,000}$ verdünnt und zwar in

E 39 $^1/_{10\,000}$, $^1/_{100\,000}$, $^1/_{1\,000\,000}$ und $^1/_{10\,000\,000}$. Zu diesem Zweck wurde eine Trockenampulle E 39 in 1,0 ml Alc. abs. aufgelöst und mit 9,0 ml Kochsalzlösung auf $^1/_{1000}$ verdünnt. Die weiteren Verdünnungen erfolgten in 1%igem Rinderalbumin (Fraction 5) in physiologischer Kochsalzlösung. Die Controllreihe ohne E 39 wurde in reiner Albumin-Kochsalzlösung suspendiert. Diese 5 Verdünnungsreihen wurden 24 Stunden bei ± 4° bewahrt und danach in der Menge von je 0,02 ml intracerebral austitriert.

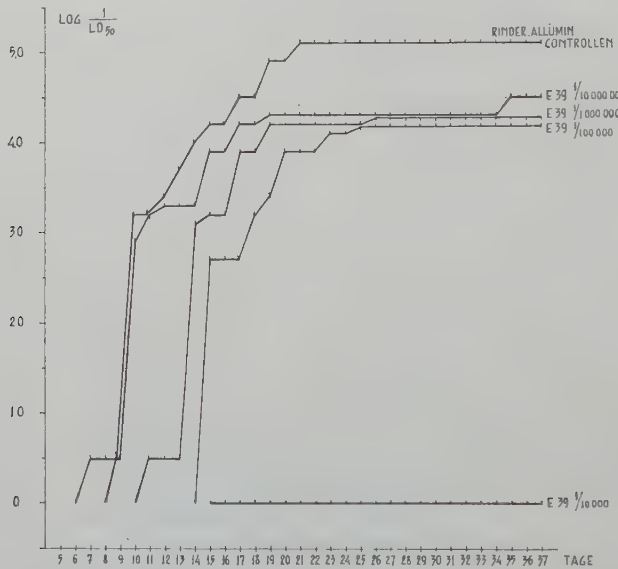

Fig. 11. Virulenzcurven von Ascites, der in verschiedenen Verdünnungen (Medium = Rinderalbumin 1%) mit E 39 24 Stunden lang bei ± 4° gehalten worden war. Verimpfung je 0,02 ml intracerebral in Serien von je 30 Mäusen.

Die Virulenzcurven sind in Figur 11 zusammengestellt. Es zeigte sich eine deutliche Virulenzverminderung, die am Ende des Versuches eine Differenz mit der Controllserie ergab, die bei der E 39-Serie von $^1/_{10\,000\,000}$ log 0,6, von $^1/_{1\,000\,000}$ log 0,8 und von $^1/_{100\,000}$ log 0,9 ausmachte. Dies entspricht einer Virulenzsenkung um das etwa 4-fache, etwa 6-fache und etwa 8-fache. Nach der Infection mit dem in E 39 $^1/_{10\,000}$ suspendierten Ascites erkrankte keine einzige Maus der Serie. Das Material hatte jegliche Virulenz verloren.

Besprechung der Ergebnisse.

Nicht nur bei der Behandlung menschlicher Tumoren, sondern auch bei Tierversuchen dürfen wir unsere Erwartungen nicht allzuhoch schrauben. Es ist gewiss wünschenswert, bei tumorinficierten behandelten Tieren eine völlige Heilung festzustellen, aber dies ist natürlich eine ideale Forderung. Vorläufig müssen wir uns zufrieden geben, überhaupt einen Einfluss des untersuchten Präparates zu sehen. Es ist aber zweifellos wichtig, nach Möglichkeit stets die quantitativen Methoden der Virologie anzuwenden, gleichgültig ob man irgend eine hypothetische Virusinfection oder die Tumorcelle als solche als Ursache der Erkrankung der Versuchstiere ansieht. Notwendig ist vor allen Dingen ein Arbeiten mit abgestuften Verdünnungen des inoculierten Materials, und dies macht die Verwendung von noch mehr Versuchstieren als bisher nötig. Aus den erhaltenen Werten lässt sich für eine constante chemotherapeutische Dosis eine Curve zusammenstellen, welche einen Eindruck von der Virulenz des Tumors im Verlauf des Versuchs ermöglicht. Ueber die Methodik haben Collier und de Wit (1960) berichtet. Am Abschluss des Versuches erhält man am Ende der Curve einen constant bleibenden LD_{50}-Wert der Versuchstiere und der Controllen, woraus sich die definitive Senkung der Virulenz infolge der chemotherapeutische Behandlung ergibt.

Remmele und Rick (1957) stellten mit E 39 vor allem Versuche in vivo an. Sie fanden, dass bei einer Dosis von 0,5 ml mit 0,01 mg ($= {}^1/_{50.000}$) die Tumoren stets angingen. Die vom Tage der Impfung ab behandelten Tiere schienen aber langsamer zu erkranken und länger zu überleben.

Bei der durch uns angewandten Technik ist diese spätere Erkrankung und längere Ueberlebensdauer in den Virulenzcurven ausgedrückt. Es hat sich eindeutig gezeigt, dass das Cytostaticum Bayer E 39 die Virulenz des Krebs-2-Ascitescarcinoms zu senken vermag. Eine zehnmal wiederholte Dosis von 0,5 ml ${}^1/_{10\,000}$ intraperitoneal senkt die Virulenz des intraperitoneal verimpften Ascitestumors auf ungefähr ${}^1/_{100}$ der normalen Virulenz. Eine einmalige Behandlung mit 0,4 ml ${}^1/_{1000}$ hatte zunächst einen deutlichen Einfluss auf die Virulenz, doch zeigte sich zum Schlusse wieder eine Zunahme bis ungefähr zur Virulenz der Controllen. Eine 4-malige Behandlung mit je 0,2 ml ${}^1/_{1000}$ senkte dagegen die Virulenz endgültig.

Interessant war auch der Vergleich der Serientherapie mit fallenden Dosen bei kurzen bzw. langen Intervallen. Bei kurzen Inter-

vallen war die Höhe der Dosierung von Einfluss auf die Virulenzsenkung, bei längeren Intervallen dagegen nicht.

Wie bereits betont, darf man nicht allzuviel von einem Chemotherapeuticum erwarten, und so ist es nicht erstaunlich, dass die intracerebrale Infection mit dem Ascitestumor therapeutisch nicht zu beeinflussen ist. In dem hier mitgeteilten Versuch war die Virulenz bei den behandelten Tieren und bei den Controllen völlig gleich.

Die Versuche einer Beeinflussung der Virulenz des Tumors bei inficiertem Tier mit Ascites in der Bauchhöhle zeigten deutlich, wie diese kräftig beeinflusst werden kann. Zu einer Heilung der behandelten Tiere kommt es nicht.

Zum Schluss ergab ein in vitro-Versuch, dass die Concentration von $^1/_{10\,000}$ E 39 nach 24 Stunden Einwirkung bei 4°C. alle Cellen völlig avirulent macht, dass aber bis zur Concentration $^1/_{10\,000\,000}$ die Virulenz erniedrigt wird.

Kein Einfluss auf die Virulenz zeigte sich bei hier nicht mitgeteilten Versuchen, in denen die intraperitoneale Infection 10-mal subcutan behandelt wurde. Dies steht im Gegensatz zu dem günstigen Einfluss bei intraperitonealer Therapie. Diese Beobachtung ist aber in Uebereinstimmung mit der verschiedentlich mitgeteilten günstigen Wirkung von E 39 bei intratumoraler Application bei menschlichen Tumoren. Die intraperitoneale Injection des Praeparates bei der intraperitonealen Infection von Mäusen ist ja in der Tat auch als „intratumorale" Therapie zu deuten.

Was die Methode der Darstellung der Versuchsergebnisse betrifft, so geht wohl aus Versuch 1 hervor, dass ein Nebeneinanderstellen der Lebensdauer der behandelten Tiere und der Controllmäuse als Colommendiagramme der verschiedenen Infectionsdosen den Wert der Therapie erkennen lässt, wenn auch das Ueberleben einzelner Tiere die Darstellung etwas erschwert. Viel eindrucksvoller ist aber doch die Gegenüberstellung der Virulenzcurven der behandelten und der Controlltiere. Die graphische Darstellung der Intervalle zwischen beiden Virulenzcurven ist aber nicht so instructiv, wie es die beiden Curven allein sind.

Zusammenfassung.

Die Anwendung von Virulenzcurven bei Versuchen über Chemotherapie bei Ascitestumoren gibt ein deutliches Bild von dem Einfluss des untersuchten Chemicals.

In Versuchen mit dem Cytostaticum ,,Bayer E 39'' beim KREBS-2-Ascites-Carcinom zeigte sich, dass bei bestimmter Versuchsanordnung eine deutliche Verminderung der Virulenz des Tumorstammes verursacht wurde. Am besten erwies sich die intratumorale (intraperitoneale) Therapie. Die intracerebrale Infection war therapeutisch nicht zu beinflussen.

Bei Versuchen in vitro war die Wirkung von E 39 auf die Virulenz besonders eindrucksvoll.

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SOME SEROLOGICAL STUDIES OF THE GENERA *CORYNEBACTERIUM*, *FLAVOBACTERIUM* AND *XANTHOMONAS* [1]

by

JOSEPH A. SODA [2] and **ROBERT C. CLEVERDON**

(Received August 10, 1959).

Diagnosis of yellow strains of *Corynebacterium*, *Flavobacterium* and *Xanthomonas* might be aided by serologic technics. This is a report of some studies regarding the serologic relationships of the somatic antigens of 21 members of these genera.

Methods and materials.

Cultures. The cultures and sources were: *Corynebacterium flaccumfaciens* (334) and *C. michiganense* (348), obtained from the U.S. Department of Agriculture; *C. rathayi* (CR1), *C. poinsettiae* (CP2) and *C. insidiosum* (C15), obtained from Dr. W. H. Burkholder's collection; *Flavobacterium acidificum* (8366), *F. arboresens* (4358), *F. esteroaromaticum* (8091), *F. flavescens* (8315), *F. suaveolens* (958), obtained from the American Type Culture Collection; *F. aquatile* (700) [3] was obtained from the Midwest Culture Service; a *Flavobacterium* sp. (designated "F60") was furnished by Dr. M. J. Pelczar, Jr.; *X. pelargonii* (8721), *X. pruni* (10017) were obtained from the American Type Culture Collection; *X. pruni* (XP13), *X. begoniae* (XB3), *X. barbariae* (XB1) and *X. campestris* (XC16) were obtained from Dr. Burkholder's collection.

Stock cultures of all flavobacteria and corynebacteria were kept

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[2]) Present address: The Upjohn Company, Kalamazoo, Michigan, U.S.A.

[3]) Regarded by Dr. O. B. Weeks as misnamed (J. Bact. **69**, **649**, **1955**).

at 4°C. on agar slants composed of: 0.5 per cent casitone, 0.5 per cent NaCl, 0.3 per cent beef extract, 0.1 per cent glucose, and 1.8 per cent agar, pH 7.2, and were transferred monthly. The xanthomonads were maintained at 4°C. on agar slants composed of: 20 per cent potato extract, 2 per cent glucose, and 1.8 per cent agar. All media used throughout the study were sterilized in the autoclave at 121°C., 15 psi, for 15 minutes and kept at room temperature for 48 hours to insure sterility.

Preparation of Antigen and Antisera. All cultures were examined for spontaneaous agglutination; those which were "rough" were subjected to repeated transfer in a wide variety of media (V-8 juice, potato agar, cabbage) or in the tube used by BRAUN and HOWELL (1947) to obtain smooth strains. Plates of agar composed of 0.5 per cent casitone, 0.5 per cent NaCl, 0.3 per cent beef extract, 0.1 per cent glucose, 1.8 per cent agar, and 0.25 per cent proteose-peptone (Difco) were swabbed with a 24 hour 'smooth' culture in casitone broth, which was reciprocally shaken at about 100 cycles per minute. After 24 hours at 28°C., the cells were harvested with 0.3 per cent formalized physiological saline, filtered through nonabsorbent cotton, washed thrice and resuspended in the formalized saline. The turbidity of the cellular suspension was adjusted to 61 per cent transmission with a Lumetron, model 400-A, using a blue filter (420 mu). The suspensions were placed in serum bottles and incubated at room temperature for 24 hours. After the sterility check was completed, they were placed in the refrigerator (4°C.) until used.

Healthy adult female rabbits, found to be free from normal antibodies for the antigen used, received 7 injections during 18 days. All intravenous injections were made in the marginal vein of the left ear. One week from the last scheduled injection, the rabbit was deprived of food for 8 hours, bled from the right ear and the following day by a cardiac puncture. The pooled serum (preserved with merthiolate 1 : 3000) was stored at 4°C.

Agglutination test. Fresh antigen was prepared as described above except that the turbidity was adjusted to 48 per cent transmission. To serial binary dilutions of the antisera, 1 ml volume in 13 mm × 100 mm test tubes, was added 1 ml of the antigen suspensions. After shaking, the tubes were placed in a water bath at 37°C., and examined at 24 and at 48 hours. All antigens and antisera were used in cross reactions.

TABLE 1.

Homologous and heterologous reactions, 5 corynebacteria, 7 flavobacteria and 7 xanthomonads

Antigen	Antiserum																		
	CR1	C334	CP2	C15	C348	F700	F8366	F8091	F958	F60	F8315	F4358	XP13	X7381	XC16	X10017	XB1	X8721	XB3
CR1	**5120***	40	0	40	0	0	0	40	0	40	80	0	40	80	0	40	0	80	0
C334	0	**320**	0	0	0	0	0	0	0	0	0	0	0	40	40	0	0	0	0
CP2	0	320	**2560**	80	0	0	80	40	0	0	80	0	80	0	0	0	0	40	0
C15	0	0	0	**640**	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0
C348	0	80	80	80	**2560**	0	0	0	0	40	80	0	80	80	160	0	80	80	160
F700	0	80	0	0	0	**1280**	80	320	320	640	320	0	40	80	0	0	0	80	0
F8366	0	80	0	0	0	40	**10240**	0	0	0	0	40	80	160	0	0	0	40	0
F8091	0	0	0	0	0	160	0	**640**	1280	320	1280	0	0	0	0	0	0	40	0
F958	40	160	80	80	0	80	80	160	**1280**	160	640	40	320	80	0	80	0	80	0
F60	0	0	40	40	80	1280	320	80	80	**640**	320	0	40	80	0	0	40	160	0
F8315	0	0	0	0	0	160	0	320	1280	320	**1280**	80	0	0	0	0	0	40	0
F4358	80	80	80	160	80	160	80	160	80	160	160	**320**	80	320	80	80	160	80	320
XP13	0	0	0	0	0	0	40	0	0	0	0	0	**20480**	0	0	5120	0	0	0
X7381	0	0	0	0	0	0	0	0	0	0	0	0	0	**640**	40	0	0	0	0
XC16	40	0	0	80	320	0	0	0	0	0	0	0	40	40	**10240**	40	0	640	80
X10017	0	0	0	0	0	0	0	0	0	0	0	0	20480	0	0	**55120**	0	0	0
XBI	0	0	0	40	160	0	0	0	0	0	0	0	40	0	5120	0	**2560**	320	160
X8721	0	0	0	40	80	0	0	0	0	0	0	0	0	0	640	0	320	**1280**	640
XB3	40	0	0	40	0	0	0	0	0	0	0	0	640	0	320	0	640	320	**5120**

* Numbers represent the denominator of greatest dilution of serum showing agglutination.
0 = no reaction at 1 : 40; homologous titres in bold face.

Results and discussion.

Table 1 shows the results of the homologous and heterologous serologic reactions, expressed as the denominator of the dilution clearly showing agglutination.

The homologous reactions of the corynebacterial antigens were of relatively high titre; their cross reactions with other corynebacterial antisera were few and of low titre, which indicates a method of differentiating these organisms.

The xanthomonad antisera were of high titre and showed high cross reactions with other xanthomonads but few outside the genus. ELROD and BRAUN (1947) have extensively studied this genus and have divided thirty-six species into 5 main groups on the basis of serologic reactions. At least one organism from each group was included in this study. In general, the limited cross reactions of the antigens with antisera from the other genera and the high homologous titres indicate the xanthomonads can be differentiated from the flavobacteria and corynebacteria.

The homologous reactions of flavobacterial antisera were of moderate titre. Cross reactions with other flavobacteria were quite numerous and in many cases of relatively high titre.

The flavobacteria were briefly studied further with the antibody absorption technic. Antiserum against F60 was chosen for these studies since it agglutinated most other flavobacteria. These results are shown in table 2.

TABLE 2.

Cross reactions of F60 antiserum absorbed with 5 flavobacteria antigens.

Absorbing Antigen Employed	Agglutinating Antigen Used				
	F 700	F 60	F 8091	F 8315	F 958
F 700	0	160*	0	160	80
F 60	0	0	0	0	0
F 8091	0	0	0	0	0
F 8315	0	0	0	0	0
F 958	160	80	80	160	0

* Numbers represent the denominator of greatest dilution of serum showing agglutination.

0 = no reaction at 1 : 40.

On the basis of these results one may postulate a provisional antigenic scheme. F60, F8091, and F8315 would be assigned an

"A" and "B" antigen; F700 only the "A" and F958 the "B". Additional antigenic sites are involved, however, since F700 antiserum also agglutinates F958. The tested flavobacteria appear to be closely allied; F700 appears to be a member of this group. Species differentiation is not corroborated by the serological findings, which suggests that the isolates may be variants of fewer or a single species.

Summary.

Antisera were prepared against members of three genera of bacteria: *Corynebacterium*, *Flavobacterium* and *Xanthomonas*. Results of cross agglutination studies indicate the organisms form three groups which follow generic lines. With the corynebacteria, low cross reactivity, and with the xanthomonads, high titre and low cross reactivity facilitate differentiating the species examined. Three of flavobacteria appear to have quite similar antigenic components so that serologic diagnosis is not realized.

References.

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Elrod, R. P. and Braun, A. C. 1947. J. Bact. **53**, 509, 519.

ISOLATION AND CHARACTERISTICS OF *TREPONEMA ZUELZERAE* NOV. SPEC., AN ANAEROBIC, FREE-LIVING SPIROCHETE

by

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A small, anaerobic spirochete, encountered in an enrichment culture of green bacteria, has been obtained in pure culture, and its morphological and physiological properties have been studied.

Isolation and maintenance.

An enrichment culture for green bacteria that can grow at the expense of an organic electron donor was prepared as follows. A bottle was filled with a medium composed of tapwater, 0.1 % Na acetate, 0.05 % Difco yeast extract, 0.1 % KH_2PO_4, 0.1 % NH_4Cl, 0.2 % $NaHCO_3$, 0.05 % $MgCl_2.6H_2O$, 0.02 % $CaCl_2$, 250μg % $FeCl_3.6H_2O$, and a trace of $Na_2S_2O_4$, adjusted to pH 7.0. After inoculation with mud from a freshwater pond, the bottle was stoppered and exposed to light of wavelength 732 mμ in order to preclude the development of algae and purple bacteria.

After a week's incubation at 28°C the medium turned green owing to the development of a *Chlorobium*-like organism. Microscopic examination revealed that the culture also contained a small spirochete, which was especially numerous in the sediment. Following a number of transfers in the above-mentioned liquid medium,

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1) Part of this work was carried out at the Hopkins Marine Station of Stanford University, Pacific Grove, U.S.A., under a Rockefeller Foundation fellowship. Present address: Laboratorium voor Microbiologie, Wageningen, the Netherlands.

during which the spirochete persisted in the cultures, agar shake cultures were prepared in a variety of media; good results were obtained with one that had the following percentage composition: KH_2PO_4, 0.1; NH_4Cl, 0.1; $MgCl_2 . 6H_2O$, 0.05; $CaCl_2$, 0.004; $Na_2S.9H_2O$, 0.05; $NaHCO_3$, 0.1; $FeCl_3.6H_2O$, 0.00025; agar, 1.5, in distilled water; pH 7.0. To 100 ml of this medium was added 0.2 ml of a trace element solution containing per 100 ml: H_3BO_3, 56 mg; $ZnSO_4.7H_2O$, 44 mg; $CoCl_2.6H_2O$, 20 mg; $CuSO_4 . 5H_2O$, 2 mg; $MnCl_2$, 2 mg; and $Na_2MoO_4.2H_2O$, 75 mg. The cultures were incubated in an illuminated incubator at 28°C.

In addition to the characteristic disc-shaped colonies of the green bacteria, the cultures contained fluffy, spherical colonies of low density, with a pronounced tendency to spread in the agar medium; these appeared to be colonies of the spirochete. Following repeated subcultures in the same medium, inoculated with suspensions from well-isolated colonies, this organism was readily isolated in pure culture. For reasons that will shortly become apparent, it was maintained in stab cultures, transferred monthly, in mineral agar with 0.2 % glucose and 0.05 % Difco yeast extract, covered with paraffin. They were incubated at 30°C. until gas production became apparent, after which they were stored at 4°C.

Nutritional requirements.

The appearance of the spirochete in the elective and shake cultures implied that it could grow in the absence of oxygen. Special experiments have shown that it is an obligate anaerobe. The following experiments were therefore conducted with cultures in completely filled, glass-stoppered bottles, or in Hall tubes (see Barker, 1936), and with media to which 0.01–0.05 % $Na_2S . 9H_2O$ was added to insure strictly anaerobic conditions.

In a mineral medium similar to that described for its isolation but lacking agar, the spirochete failed to grow. Supplementation of the medium with 0.2 % glucose and some Difco yeast extract provided conditions for copious cultures; 0.02–0.05 % yeast extract suffices for maximal development. Good growth was also obtained if the yeast extract was replaced by 0.2 % Bacto tryptose, tryptone, peptone, proteose peptone, or casitone. No growth occurred in these media unless sugar was also present. Casamino acids, even if not vitamin-free, did not serve as a substitute for yeast extract; nor did

a mixture of 21 amino acids, 3 purines, and 8 vitamins, as used in the synthetic medium for lactic acid bacteria and described in the National Dairy Res. Lab. Manual (1949; see THIMANN, 1955, p. 425). The results remained negative after enriching the latter medium with α-lipoic acid, vitamin B_{12} and heme (final concentrations 100, 20 and 1000 micrograms per 100 ml respectively).

From these results it may be concluded that the growth in the mineral agar medium from which the spirochete was isolated must be attributed to the presence of impurities in the agar; a comparable behaviour has been encountered by BACHMANN (1955) during her studies with *Cytophaga fermentans*.

Comparison on the basis of turbidity, and gas and acid production in cultures with diverse sugars, revealed that glucose, mannose, galactose, arabinose, xylose, trehalose, cellobiose, maltose, and starch can be used by the spirochete as fermentable substrates; and, although the difference was but slight, maltose yielded consistently better growth than glucose. Fructose, sorbose, rhamnose, sucrose, lactose, raffinose, inulin, mannitol, and sorbitol are not fermented, and do not permit growth of the spirochete.

The optimum pH for growth is 6.8–7.8; the sugar decomposition is accompanied by acid and gas production, and growth ceases when the pH has dropped below 6. The addition of bicarbonate (0.1–0.5%) to the medium maintains the reaction within the favourable range if the sugar concentration does not exceed 0.2 %. But no growth occurs if the bicarbonate is replaced by appropriate phosphate buffers; evidently the organism has an absolute requirement for CO_2.

The optimum temperature is 37–39°C; good growth takes place over the range of 20–40°C., but not any longer at 45°C.

Colonies in agar cultures show a marked difference in appearance, dependent on the composition of the medium. The large spreading colonies, which are characteristic for cultures in ,,mineral'' agar and whose diameter in sparsely seeded tubes may eventually attain to that of the culture tube, are never found in glucose-yeast agar, where the diameter does not exceed a few millimeters. The spreading type of growth has also been observed by BRYANT (1952) in cultures of an anaerobic spirochete, isolated from bovine rumen, in roll tubes with cellulose-rumenfluid agar, where the spirochete accumulates at the periphery of clear areas around colonies of cellulolytic micro-organisms.

The pattern of glucose fermentation.

Virtually nothing is known about the biochemical activities of spirochetes. The availability of a pure culture of an anaerobic representative thus provided an excellent opportunity to study the fermentation products formed during the anaerobic decomposition of glucose.

A. Apparatus.

The fermentation was studied with the apparatus shown in Fig. 1. Because it is convenient and generally useful for such studies, a brief description follows.

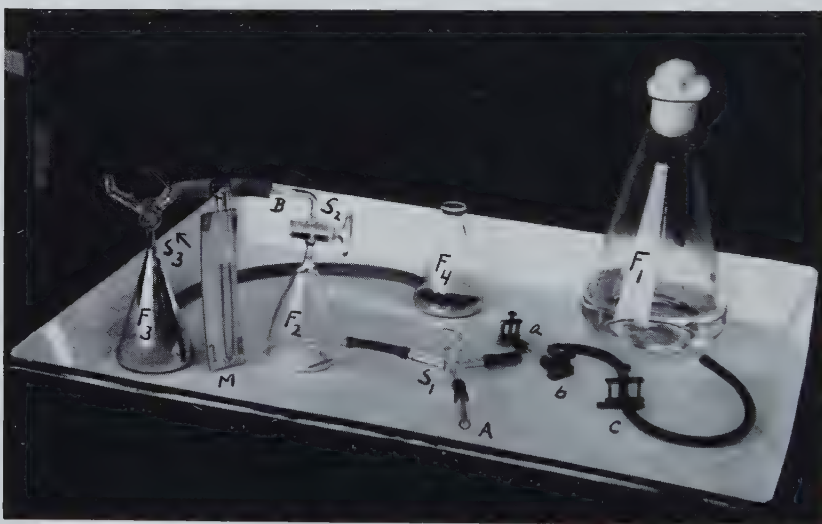


Fig. 1. Fermentation apparatus. Explanation see text.

A one-liter Erlenmeyer flask, F_1, containing the culture medium without sugar, $NaHCO_3$, and Na_2S, is connected by means of rubber tubing to a 100 ml fermentation flask, F_2. Screw clamp *c* prevents the entrance of the medium into F_2. Cotton plugs are inserted at A and B, and the assemblage is sterilized by autoclaving. If silicone high-vacuum grease is used for stopcocks S_1 and S_2, these need not be autoclaved separately.

After sterilization the medium is cooled rapidly, and F_2 is connected with the remainder of the apparatus, consisting of a manometer, M, flask F_3 completely filled with mercury and provided with

a three-way stopcock, S_3, and the leveling flask, F_4, attached to F_3. Separately sterilized glucose, $NaHCO_3$, and Na_2S are added in requisite amounts to the medium in F_1; at the appropriate stage this medium is inoculated. Next, clamp *c* is opened so that the medium fills the apparatus as far as the three-way stopcock, S_1. The air in F_2 is replaced by N_2, introduced at A. The closed arm of the manometer is filled with N_2 by raising and lowering the Hg level in this arm (by changing pressure on open arm) while F_2 is flushed with N_2. Finally, about 80 ml of the medium is pressed into F_2 by lifting F_1; stopcock S_1 is then turned so that F_1 can be detached; S_3 is turned to connect F_3 with F_2. During the fermentation the pressure in F_2 is maintained at one atmosphere by means of F_4.

Only one fermentation flask is shown in the figure; as a rule, an additional one, attached to *a*, is used which is filled with the medium before it has been inoculated, and thus serves as a blank for determining initial glucose and CO_2; another flask may be added at *b*, which then is also connected with a flask similar to F_3 to which a leveling flask is attached.

B. Methods of analysis.

After completion of the fermentation, water is added to F_2 in order to drive all the gas into F_3, and the amount of water, introduced with a hypodermic syringe injected into the rubber tube between S_1 and F_2, is measured. F_2 is then disconnected after closing S_2. The gas collected in F_3 is analyzed in a manometric gas analysis apparatus, where CO_2 is absorbed by alkali, and H_2 burned by passage over CuO at 800°C.

About 50 ml of culture liquid is then collected from F_2 for analysis of fermentation products. The remaining liquid is used to determine dissolved CO_2. F_2 is connected at B with a CO_2 train and 1.5 ml of 6N H_2SO_4 added with a hypodermic syringe through rubber tube between flask and S_1. Then CO_2-free air is passed through the acidified culture liquid, entering the flask through a hypodermic needle, injected as above. The gas is carried through a Drechsel gas-washing bottle with concentrated H_2SO_4 to remove moisture and subsequently CO_2 is collected in ascarite. The determination of CO_2 dissolved in the culture medium can also conveniently be carried out with the aid of a van Slyke apparatus.

The fermented medium is systematically analyzed for residual sugar, volatile and non-volatile neutral products and acids,

after precipitating the protein with $Zn(OH)_2$ (NEISH, 1952).

For the present investigation the following specific methods were used. Glucose was determined by the method of Luff, as described by SCHOORL (1929). Volatile acids, collected by steam distillation, were qualitatively identified and quantitatively estimated by the method of FRIEDEMANN (1938); redistillation of the steam distillate in the presence of HgO served to test for the presence of formic acid. Ether extracts of acidified steam distillates were also analyzed by paper chromatography according to KENNEDY and BARKER (1951). Lactic acid was determined by the method of FRIEDEMANN and GRAESER (1933), succinic acid with the aid of succinic acid dehydrogenase by the method of KREBS as described by COHEN (See UMBREIT, BURRIS and STAUFFER, 1957). Fermentation acids were also identified and estimated by the silica gel partition chromatographic method (NEISH, 1952), using a fraction collector as described by LENS and EVERTZEN (1952), and silica gel prepared by the method of NIJKAMP (1954).

Distillates of alkalinized aliquots of the fermented medium were used to determine alcohols by the dichromate method described by NEISH (1952). Tests for glycerol, 2,3-butane diol, acetoin, and diacetyl were performed by NEISH's (1952) periodate method, modified by introducing a saturated solution of 2,4-dinitrophenyl hydrazine in 2N HCl in the center well of the Conway diffusion unit. The tests were carried out on deproteinized samples of culture liquid which did not contain residual sugar.

C. Results.

The analyses have shown that the fermentation products are composed of H_2, CO_2, and acetic, lactic, and succinic acids. These account for over 90 % of the fermented sugar. Formic acid, ethanol, and glycols have not been encountered.

The fermentation generally ceases when about 0.2 % glucose has been fermented; hence higher sugar concentrations have not been used.

The rate of gas production was markedly enhanced by the addition of a few mgs of diatomaceous earth (Celite No. 535) to 100 ml of medium; the ratio in which the end products are formed is not influenced thereby.

Results of two representative experiments, with media containing 0.1 and 0.5 % $NaHCO_3$, respectively, are collected in Table 1.

It will be seen that the fermentation pattern of the spirochete

TABLE 1.

Glucose fermentation in media containing 0.1 and 0.5 % $NaHCO_3$.
Results expressed as millimoles product/millimole glucose fermented.

Fermentation products	0.1 % $NaHCO_3$	0.5 % $NaHCO_3$
Lactic acid	0.79	0.87
Acetic acid	1.10	0.82
Succinic acid	Trace	0.13
Carbon dioxide	0.97	0.68
Hydrogen	1.82	1.64
Carbon recovery, %	92.3	90.8
Redox index	1.06	0.90
Ratio CO_2/H_2	0.53	0.41

resembles that of *Escherichia coli,* although it differs in that the latter organism produces ethanol and relatively much less hydrogen. It is also rather similar to that of the anaerobic rumen spirochete studied by BRYANT (1952). But the latter organism does not produce any H_2, and ethanol represents one of its major fermentation products.

Since the amount of succinic acid produced appears to be a function of the CO_2 concentration of the medium, it is probable that this acid arises from a CO_2 addition to a three-carbon compound. ELSDEN (1938), who discovered a similar effect on the sugar fermentation by *E. coli,* advanced this hypothesis long ago; it was later verified by tracer studies. The occurrence of such a mechanism in our spirochete is supported by the fact that in the two fermentations represented in the Table the number of moles of CO_2 and succinate combined is approximately the same. Also the ratio of CO_2 to acetate more nearly approaches unity if succinate is included as a CO_2 derivative.

The large amount of H_2 obviously cannot be formed entirely by a decomposition of formic acid, which would require a CO_2/H_2 ratio of 1. It is therefore necessary to invoke at least an additional mechanism for H_2 production.

Whether the end products originate from a primary fission of the sugar to two three-carbon moieties, or arise by a degradation via gluconic and ketogluconic acid, cannot yet be decided. Although the strictly anaerobic nature of the spirochete would seem to favour the former alternative, studies on the decomposition of presumed

intermediate products and precursors, eventually with labeled substances, are required; these are being contemplated.

Morphology.

The spirochetal cell is characterized by being flexible, spiral shaped and able to perform a corkscrew-like motion. Moreover, a particular characteristic, specific for spirochetes, is the occurrence of an axial filament (axistyle), consisting of one or more fibrils, and wound spirally around the protoplasmic cylinder from end to end. This gives the organism the appearance of a two-strand flex.

Fig. 2. Electron micrograph of spiral form; 36000 ×.

Fig. 2 shows an electron micrograph of our spirochete; the three components which have also been encountered in other spirochetes, *viz.*, a coiled protoplasmic cylinder, a cell membrane, and an axial filament, are clearly evident.

Czekalowski and Eaves (1955) treated cells of *Leptospira* with low concentrations of sodium desoxycholate for short periods of time and found that this treatment results in the axistyle becoming

separated from the coiled protoplasmic cylinder. After its release the axistyle remains attached to the ends of the protoplasmic cylinder, suggesting that it is more firmly connected with the ends than with the rest of the cell.

In fact, the first evidence for the existence of an axistyle in spirochetes was obtained by early investigators (cf. NOGUCHI, 1928) who treated cells with bile or bile salts and found that prolonged treatment caused the protoplasm completely to disintegrate, while the more resistant axial filament remained intact. Although the existence of the axistyle in at least several genera had thus been proved, its relation to the spirochetal cell could not be revealed until the introduction of electron microscopic observations. Electron micrographs have revealed an axial filament, wound around the coiled protoplasmic cylinder in *Treponema* (SWAIN, 1955; MÖLBERT, 1956 a), *Borrelia* (SWAIN, 1955; MÖLBERT, 1956 b), *Leptospira* (e.g. BABUDIERI, 1949; MÖLBERT, 1955; SWAIN, 1957) and *Cristispira* (BRADFIELD and CATER, 1952), while ZUELZER (1910, 1931) and NOGUCHI (1928) have presented evidence for the presence of an axistyle in *Spirochaeta plicatilis*. The contrary findings reported by DYAR (1947) do not negate the validity of the earlier work because the culture she studied was not a spirochete at all (PRINGSHEIM, 1949).

The number of fibrils in the axial filament varies from one (SWAIN, 1955, 1957) or 5–6 (MÖLBERT, 1955) in *Leptospira* to more than a hundred in *Cristispira* (BRADFIELD and CATER, 1952). Unfortunately the number of fibrils in the axistyle of our spirochete cannot be determined from the only electron micrograph available at present (fig. 2). Although it seems that the axistyle in this case is inside the cell membrane, further confirmation is needed. In some spirochetes the axial filament appears to be on the outside of the cell membrane (e.g. *Cristispira*; BRADFIELD and CATER, 1952), whereas in others it has been found inside this structure (e.g. *Treponema*; MÖLBERT, 1956 a). Fig. 2 suggests that the latter situation also applies to our spirochete.

This figure fails to show evidence for the presence of flagella, which is in line with the observations of BRADFIELD and CATER (1952) who never observed such organelles in electron micrographs of carefully handled spirochete preparations. On the other hand, it has been claimed that spirochetes do possess flagella (e.g., MORTON *et al.*, 1951; WATSON *et al.*, 1951). But this interpretation has been challenged by SWAIN (1955) and MÖLBERT (1956 b), who contend

that the presumed flagella are actually dislodged fibrils of the easily damaged axistyle.

The purported demonstration of flagella in spirochetes by staining techniques (Leifson, 1950) must also be regarded with reservations.

Bradfield and Cater (1952) demonstrated by a simple model that the axial filament may act as an elastic band, which confers on the spirochete its spiral form, and presented evidence that the fibrils in the bundle are contractile. Additional evidence has been obtained by Swain (1955) who showed that treponemata, whose fibril bundles had been ruptured or displaced, flatten out.

Though it seems likely that interaction of the elastic protoplasmic cylinder and a contractile fibril bundle is responsible for the movements of the spirochetal cell, as suggested by several authors, the details of the mechanism of locomotion still remain to be worked out. Chemical analysis of cytoplasm and axistyle has shown that the latter contains more basic amino acids; DNA appears to be localized mainly in the protoplasm, whereas RNA is equally distributed between protoplasm and axial filament (Siefert, 1958).

As early as 1928, Noguchi noticed the presence of an end filament in *Treponema* and *Leptospira*. Mölbert (1956 b) has published an electron micrograph of *Borrelia recurrentis*, showing an end filament, probably formed by both cell membrane and axial filament. An end filament has also been encountered in a number of cells of our spirochete as revealed by the phase contrast microscope (fig. 4–1). Our findings are in agreement with those of Mölbert (1956 b) who found only a few cells exhibiting this filament; and it seems likely that it is formed from remnants of cell membranes and axial filaments, which have no actual function, and which adhere to some of the cells after cell division.

Developmental history.

The development of cultures of our spirochete was studied with the aid of a Wild phase contrast microscope as follows. To 10 ml of an agar medium, containing only 0.01 % glucose to limit gas formation, and cooled to 45°C after sterilization, a few drops of a 2-day old culture from a Hall-tube were added. After mixing, a small amount of the inoculated agar was placed on a sterile depression slide; a sterile cover glass was placed on top of the agar so that no air bubbles were trapped. The edges of the cover glass were sealed with paraffin, and the slide placed in an electrically heated

bottomless brass chamber attached to the mechanical stage of the microscope. The slide thus forms the bottom of the chamber; on top of it was placed a perspex plate with a perforation for the objective. The slide cultures were generally incubated at 37°C.

Just below the cover glass a thin liquid layer often develops; thus the movements of the spirochetes in liquid as well as in the agar could be studied.

At 20°C most cells are not very active, but at 30–40°C the cells in the liquid layer show the typical corkscrew-like motion. Lashing movements, in which the cells display a remarkable degree of flexibility, can readily be observed. Organisms embedded in the agar also move about actively, and again show a pronounced flexibility.

The cells multiply by transverse fission. Organisms about to divide show a constriction at or near the middle of the cell. During the process of cell division the cells are very active. The two cells connected by a narrow strand may show coordinated rotating movements, but generally the movements are not coordinated. For instance, one cell may loosely adhere over its entire length to the cover glass, carrying out lashing movements, whereas the second cell, whose axis is about perpendicular to that of the first, carries out violent rotating movements. Sometimes such a situation leads to fission, but generally the cells do not remain attached to the cover glass for a long time and restlessly change to another position.

It may happen that one cell makes a 180° turn around the point of constriction and becomes entwined with the other, both cells then twirling around in the liquid. This phenomenon is probably responsible for statements of longitudinal cell division reported in the older literature on spirochetes.

Cells which are more or less entwined often show flexing movements by which they twist apart.

On several occasions it has been noticed that, after a long time of ,,struggling'' in the thin liquid film, two cells penetrate into the agar where soon afterwards cell division occurs. It seems that flexing movements, aided by the mechanical resistance provided by the agar, cause a more rapid break of the connecting strand.

The generation time during the exponential growth phase in our slide cultures at 37°C was measured by counting the cells in agar columns of a depth of 200 μ and a diameter of 180 μ; it appeared to be about 3–4 hours. This value is close to that observed by SCHMEROLD (1956), who found a generation time of two hours in

cultures of the Reiter spirochete, cultivated in a suitable medium under optimal growth conditions. Rose and Morton (1952) mention an average generation time of 6.9 hours for the first 48 hours of growth in a culture of the Reiter spirochete. As this probably includes a lag phase, a shorter generation time during the exponential phase is likely. Thus, neither in our own experiments nor in those of other investigators generation times shorter than several hours have been encountered in spirochetes belonging to the family *Treponemataceae.*

Immediately after cell division the average length of our spirochete is 8 μ (fig. 4–2); the coils of the organism have an average wavelength of 1.1 μ. The width of the cells is 0.19–0.35 μ. Fig. 4–3 shows a cell just before division.

During the late stationary phase very small (fig. 4–4) as well as very long cells (fig. 4–5) may be found. The long cells generally show a number of constrictions, suggesting that they are composed of more than one individual. For instance, a 77 μ long cell with 9 constrictions has been observed. Were the constrictions evidence of not fully separated individuals, each cell would have a length (average) of 7.7 μ, which compares well with the normal size.

In still older cultures the constrictions become less conspicuous and the coils more irregular. Most cells become immotile towards the end of the stationary phase.

Particular attention has been paid to the formation of spherical bodies in the slide cultures. Especially during the stationary phase many cells show such structures, generally, though not always (fig. 4–6) located at the end of the cell (fig. 4–7). The spiral cell gradually becomes enveloped by the round body (fig. 4–8); eventually the coiled filament is completely enwrapped, so that the cell becomes a sphere in which the filament is still visible. The electron micrograph reproduced in Fig. 3 clearly shows such a structure.

Occasionally a very short filament may be seen inside a globule where it exhibits Brownian movements which often last for many days (fig. 4–9).

The diameter of spherical bodies in which a filament is visible generally does not exceed 3–4 μ.

It has repeatedly been noticed that a globule gradually increases in diameter, and that the spiral filament attached to it straightens out and forms one or more additional globules (fig. 4–10, 11); sometimes the filament breaks up into many small spherical bodies (fig. 4–12).

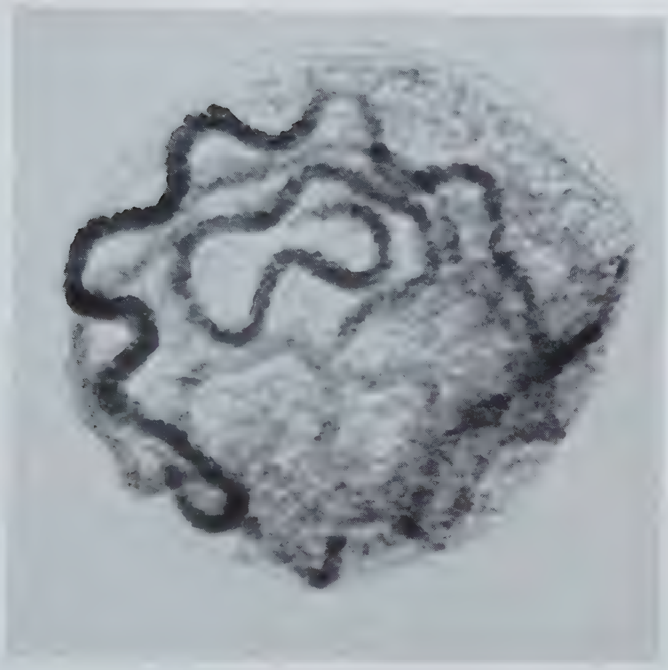


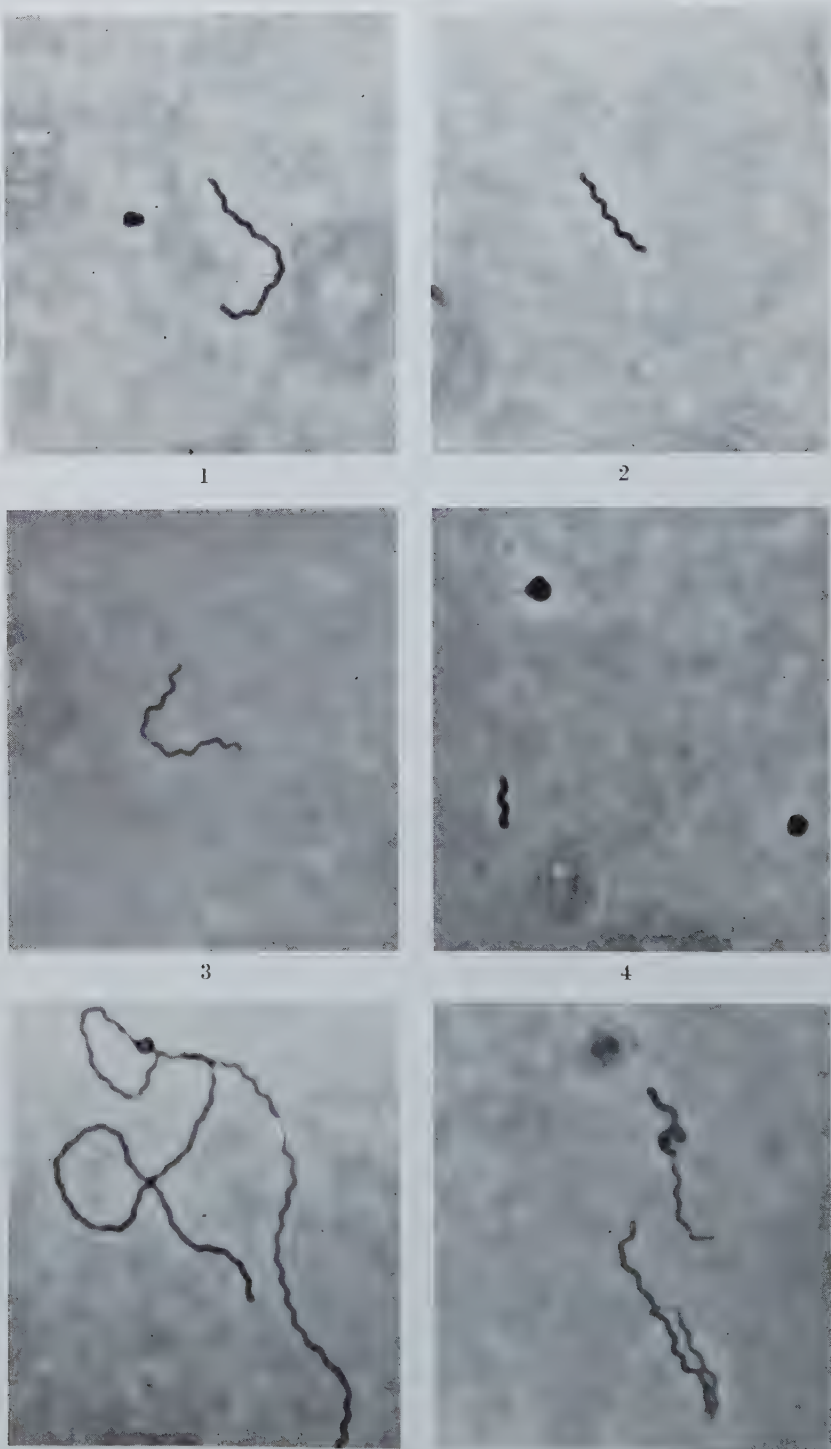
Fig. 3. Electron micrograph of granule enveloping coiled cell; 26500 ×. Preparations for electron photomicrography were prepared by Dr. A. L. Houwink (Delft) as follows. A drop of medium was brought on a collodion filmed grid and subsequently washed with distilled water. The preparation was then allowed to dry at room temperature and shadowed with platinum. No fixative was used.

The increase in size may also occur in globules in which the filament has been completely absorbed. In this case too the filament generally separates into small, dense, round bodies (fig. 4–13, 14), although globules with a single dense spherical body have also been found.

Some of the transformations described above may occur inside 15 minutes; but frequently many hours are required for its completion.

The aberrant forms described above are formed not only in slide cultures; they also develop regularly in liquid media.

In liquid cultures incubated for 4 days or longer the ratio of abnormal to normal cells is about 4 : 1. If such a culture is used as inoculum for a slide culture, this ratio can be seen to change in favour of normal cells. Towards the end of the exponential growth phase, which is reached after about 48 hours' incubation, the ratio



1 2 3 4 5 6

Fig. 4.

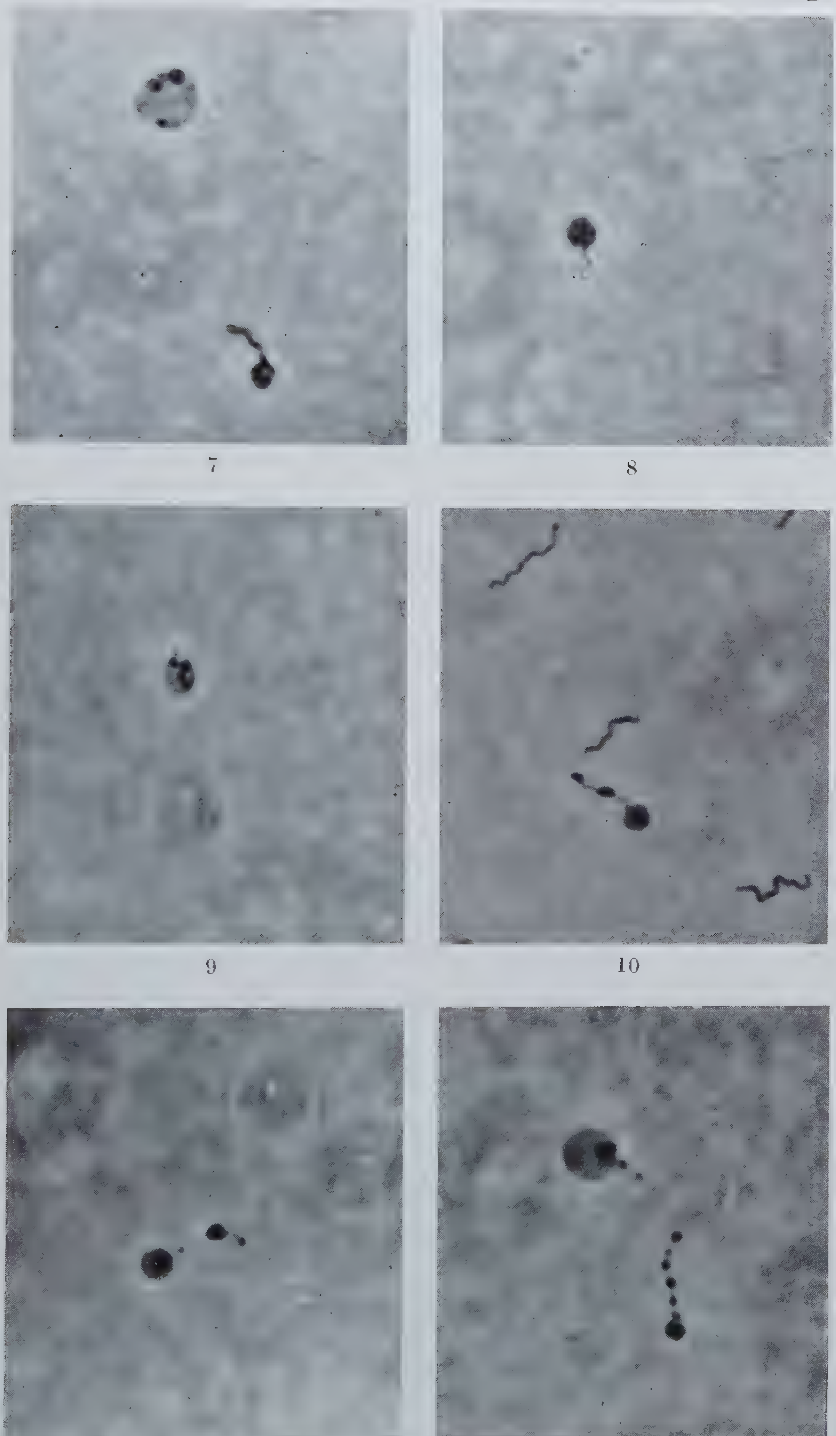
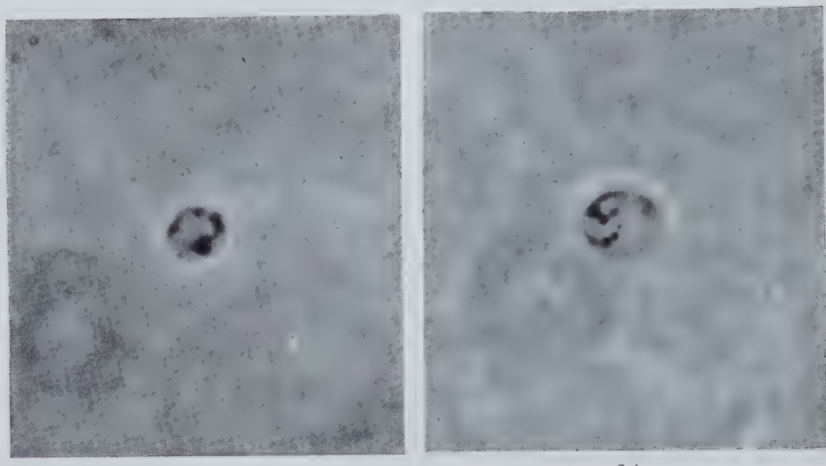


Fig. 4.



13 14

Fig. 4, 1—14. Photomicrographs made of different organisms in a slide culture after 4 days' incubation at 37°C. All forms shown occurred simultaneously in same culture. A small number of motile cells was still present. Phase contrast; 1625 ×.

of abnormal to normal cells is about 1 : 14. Then, as cell division ceases, an increasing number of spirochetes become transformed into spherical bodies; at the end of 4 days there are again about 4 times as many abnormal as normal cells.

Special attention has been paid to the behaviour of spherical forms suspended in a fresh agar medium. Many cells in such a preparation were mapped and their fate followed until the stationary phase was reached. Of the hundreds of abnormal cells thus watched, none of the large globules containing the small, dense spherical bodies, nor of the smaller round structures in which a spiral filament can still be discerned has ever been found to yield normal spirochetes, whereas during this time the normal cells multiply, and the formation of small gas bubbles in the culture indicates that glucose is fermented during the exponential growth phase. Thus experiments to date have failed to show that the abnormal cell forms are viable and represent some stage in a normal life cycle. Nevertheless, it would seem premature to conclude that they are not; it is conceivable that some kind of physical or chemical shock may be required to cause them to develop.

The formation of spherical bodies more or less similar to those found in spirochetes also occurs in cultures of *Spirillum lunatum.* Williams and Rittenberg (1956) have provided convincing

evidence that they can germinate and give rise to normal cells.

The formation of spherules in cultures of spirochetes has been the subject of much speculation, but relatively little experimentation. The phenomenon has been observed in organisms belonging to the genera *Leptospira* (e.g. SWAIN, 1955; GÄNGEL and THEMANN, 1956), *Borrelia* (e.g. HAMPP *et al.*, 1948; SWAIN, 1955), and *Treponema* (e.g. DELAMATER *et al.*, 1951, ROSE and MORTON, 1952). BRYANT (1952) also encountered it in cultures of the anaerobic spirochete he isolated from bovine rumen.

In his critical review of the literature pertaining to the formation and significance of spherical bodies in cultures of *Treponema pallidum*, INGRAHAM (1932) concluded that the interpretation of these structures as resistant (infective?) stages of the organism needs confirmation. Although he did not summarily dismiss this possibility, he emphasized that the claims were not supported by unequivocal experimental results. Later investigations have not changed this situation (STAVITSKY, 1948).

Samples containing 3–15 spherical bodies were succesfully transferred from aged cultures of *Leptospira biflexa* by BESSEMANS *et al.* (1942), using a micromanipulator. These cultures consisted of a mixture of abnormal and normal cells.

Spherical cells of *Treponema pallidum* were inoculated into rabbits by BESSEMANS *et al.* (1947) by means of micromanipulation and appeared to be non-infective, whereas normal cells, transferred in the same way, were infective.

HAMPP (1951) made successful transfers of old cultures of oral treponemes which presumably contained only round bodies. In similar studies, BRYANT (1952), and ROSE and MORTON (1952) made transfers from old cultures containing considerably more spherical than spiral forms, and compared the number of viable units in relation to the numbers of spherules and spiral forms in the inoculum. Their results did not give conclusive evidence as to the viability of granules.

In a series of eight articles, entitled ,,Studies on the life cycle of spirochetes'', DELAMATER and coworkers (1950–1951) reproduced many photomicrographs in support of the thesis that the round bodies can develop into normal cells. It must be granted that, from the point of view of photography, the documentary material is of very high quality. Nevertheless, it does not suffice to substantiate the contention, which is based entirely on the reconstruction of a

life cycle from micrographs of different cells, rather than resting on continuous observations of single individuals and their progressive developmental stages.

To prove the existence of a life cycle "it is necessary to reproduce at will the passage of one form into another under defined experimental conditions" (Dubos 1945, p. 184). As yet this proof is lacking with regard to spirochetes.

Classification.

At present the spirochetes are subdivided into the two families, *Spirochaetaceae* and *Treponemataceae* (Breed *et al.*, 1957). The small size of our organism rules out its allocation to the former, while its properties are consistent with those of the latter group, which comprises the genera *Borrelia*, *Treponema*, and *Leptospira*. Assignment to the lastnamed taxon is precluded because our spirochete is a strict anaerobe; this leaves a choice between *Borrelia* and *Treponema*.

The differentiation of these genera is based largely on staining properties; in contrast to *Treponema*, *Borrelia* species are reported to stain readily with ordinary aniline dyes. This difference may well be a function of the relative thickness of the organisms (Noguchi, 1928), which also accounts for differences in visibility by conventional light microscopy. From electron micrographs Swain (1955) has measured the diameters of *Borrelia* and of *Treponema* species as 0.24–0.60 and 0.09–0.18 μ, respectively. Babudieri (1949) has rightly pointed out that *Leptospira* "cannot be seen in the normal optical microscope, not because, as is generally thought, the refractive index of its protoplasm is equal to that of water, but because its transverse diameter is smaller than half the average length of the light wave (0.5 micron), and therefore is beyond the limits of resolution of the optical microscope". This applies equally to *Treponema*.

Another morphological feature used to distinguish *Borrelia* from *Treponema* is the width of the coils, reputed to be somewhat greater for the former. Finally, *Borrelia* species are principally blood-, treponemata tissue parasites.

That the demarcation of the two genera is far from sharp becomes evident if one tries to classify a culture such as the spirochete which is the subject of this paper. Its diameter is 0.19–0.35 μ; it is nearly invisible by ordinary light microscopy; easily stained with basic

fuchsin, carbol-fuchsin, and carbol-erythrosine, poorly with crystal violet and methylene blue, and not at all with erythrosine, safranin, malachite green, and Bismarck brown. Its morphological properties are therefore intermediate between those of the typical *Borrelia* and *Treponema* species, which makes it difficult to assign it to either genus.

Moreover, while all the hitherto recognized *Borrelia* and *Treponema* species are parasites, our isolate is non-pathogenic for mice, rats, and guinea pigs. Subcutaneous and intraperitoneal injection with healthy yeast extract-glucose cultures did not produce any abnormal symptoms during the several months during which the animals were kept under observation. And blood samples, taken 1–14 days after injection, did not reveal the presence of spirochetes by microscopy, nor by inoculation into yeast extract-glucose media.

Its nutritional requirements, too, differ markedly from those of the parasitic spirochetes. As mentioned earlier, it can be grown in a mineral agar medium at the expense of organic impurities in the agar. None of the *Borrelia* and *Treponema* species can develop in such a medium; they even fail to grow in yeast extract-glucose, in which our culture multiplies profusely. A similar differential behavior is shown by the representatives of the genus *Leptospira*; its saprophytic species can readily be differentiated from the parasitic ones by the ability of the former to grow in simple media.

The studies of Zuelzer (1931, 1936) indicate that the autochthonous population of sulfide-containing marine and freshwater muds comprises a variety of "kleinen und kleinsten Wasserspirochäten vom pallida-, recurrens-, refringens-, buccalis- und icterogenes Typus und allen möglichen Uebergänge dazwischen, meist so ausserordentlich schwach lichtbrechend, dass sie im durchfällenden Lichte im Mikroskope meist gar nicht, sondern oft nur im Dunkelfelde erkennbar sind; sie sind ausserordentlich schwer färbbar" (1931, p. 1668). The remark about the existence of "all possible intermediate stages" indicates that a subdivision based on mere differences in size, shape, and attendant staining properties is apt to be unsatisfactory. The same conclusion was voiced by Noguchi (1928), who considered a separation of *Borrelia* and *Treponema* undesirable since "the variation between *recurrentis* and *refringens* is hardly greater than that between *refringens* and *pallidum* and is only a matter of the thickness of the protoplasmic coat", and because "under cultural conditions, as the writer observed in 1913, the only difference actually existing between *B. refringens* and

T. pallidum is in staining property, the refringens having more protoplasmic substance and therefore taking stains more readily". It is also supported by my own comparison of the pathogenic and Reiter strains of *T. pallidum*, showing that the latter is coarser and more irregular than the former, and of slides in the collection of the Laboratory of Tropical Medicine at the University of Leiden, from which it appeared that it is well-nigh impossible to distinguish between Giemsa-stained *T. pertenue* and *B. duttonii*.

In view of the results of serological experiments it seems expedient to assign our spirochete to the genus *Treponema*. These experiments, which were recently carried out by DE BRUIJN (Utrecht), indicate that our organism is serologically related to *Treponema pallidum*.

A cell-free extract of our spirochete was treated with solutions of $(NH_4)_2SO_4$ (up to 100% sat.) as described by DE BRUIJN (1957). The precipitate was centrifuged, dissolved in saline buffered with 0.0045 M veronal, dialysed until free of $(NH_4)_2SO_4$ and preserved with „Merthiolate" (1 : 10000).

The solution thus obtained showed a positive complement fixation reaction with human syphilitic serum; no complement fixation was observed, however, with serum of mice which had been infected with *Borrelia duttonii*. The positive reaction with syphilitic serum appeared to be due to the presence of a protein antigen in our organism.

A detailed account of the serological experiments will be published in this journal. At present they provide the most reliable basis for the generic designation of our spirochete.

In honor of Dr. MARGARETE ZUELZER, whose studies have called attention to the group of free-living water spirochetes, the specific name *zuelzerae* is proposed.

The difficulty of making a choice between *Borrelia* and *Treponema* could have been circumvented by creating a new genus for the small, anaerobic, saprophytic spirochetes. However, since only one representative has thus far been studied in pure culture, it seems preferable to postpone such an approach until a comprehensive comparative study of this group has provided a solid foundation for rational subdivision.

Description of *Treponema zuelzerae*.

Flexible cells, 0.19–0.35 by 8–16 μ, with rather flat, irregular coils; spiral amplitude about 1 μ. Occasionally 2–3 μ long cells are

found; in old cultures long elements, up to 80 μ, resulting from incomplete division, occur. The structure of the cells is coarser and more irregular than that of "typical" *Treponema* cells. Spherical elements are formed during the stationary phase of a culture, usually at the end of the cells. Motile with an active spinning motion. Lashing movements are common. Nearly invisible with ordinary illumination.

Stain intensively with basic fuchsin, carbol-fuchsin, and carbol-erythrosin; poorly with crystal violet and methylene blue; not at all with erythrosin, safranin, malachite green, and Bismarck brown.

Growth in agar greyish, spreading, of low density; colonies usually spherical, sometimes disc-shaped.

Glucose, mannose, galactose, arabinose, xylose, trehalose, cellobiose, maltose, and starch are fermented; from glucose carbon dioxide and hydrogen, and acetic, lactic, and succinic acids are formed. Fructose, sorbose, rhamnose, sucrose, lactose, raffinose, inulin, mannitol, and sorbitol are not fermented.

Ammonium salts or nitrates cannot serve as sole nitrogen sources; yeast extract, tryptone, peptone, and casitone are adequate.

Carbon dioxide is an absolute requirement for growth.

Sulfide is not produced from sulfate.

Optimum temperature 37–40°C; grows well at 20°C., not at 45°C.

Optimum pH 7–8; fermentation ceases at pH 6.

Strictly anaerobic.

Not pathogenic for laboratory animals.

Antigenic character: possesses protein antigen that gives positive complement fixation reaction with syphilitic serum.

Isolated from freshwater mud containing H_2S.

Habitat: mud.

Cultures of *Treponema zuelzerae* have been deposited in the collection of the Microbiological Laboratory of the Technological University, Delft, and in the National Collection of Industrial Bacteria, National Chemical Laboratory, Teddington, Middlesex.

Summary.

An anaerobic, free-living spirochete was isolated from mud. The organism can be cultivated in ordinary nutrient media, e.g. yeast extract-glucose. End products of glucose fermentation are: lactic, acetic, and succinic acids, CO_2, and H_2.

In cultures of this organism spheroid bodies are formed, espe-

cially during the stationary growth phase. Studies of slide cultures showed that these bodies, when inoculated in fresh medium, do not give rise to spiral cells whereas a rapid multiplication of normal cells, also present in the inoculum, was observed.

Since the organism is serologically related to *Treponema pallidum,* it has been assigned to the genus *Treponema,* and is here described as *Treponema zuelzerae* nov. spec.

Acknowledgements.

I am grateful to Professor C. B. van Niel for his continuous interest, valuable advice and for assistance in the preparation of the manuscript. My thanks are due to Dr. A. L. Houwink who operated the electron microscope and to Professors N.H. Swellengrebel, P.H. van Thiel and J. W. Wolff for valuable discussions about parasitic spirochetes.

Dr. J. J. Laarman kindly carried out the experiments on pathogenicity and demonstrated a culture of *Borrelia duttonii.* Dr. J. H. de Bruijn graciously provided suspensions of the pathogenic and Reiter strain of *Treponema pallidum* and carried out the serological experiments. It is a pleasure to acknowledge the assistance of Miss A. van Mourik.

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FIXATION OF THE AGGLUTINOGEN FROM *HEMOPHILUS PERTUSSIS* ON THE BACTERIAL SURFACE

by

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(Received October 15, 1959).

Introduction.

When we tried to prepare a stable coloured antigen from *Hemophilus pertussis* we made the remarkable observation that with a number of methods the agglutinogen is almost lost from the bacterial surface. As this antigen is rather important in vaccine production and several methods are described for liberation of this antigen from the bacteria (see e.g. Flosdorf and Kimball, 1940) we thought it could be of value for other workers in this field to describe our observations.

Experimental.

For the preparation of the coloured antigen described in our previous communication (de Bock and Worst-van Dam, 1960), we tried the following methods.

The suspension of bacteria was always made as described before, but instead of resuspension of the compact precipitate in

A: saline with 0.5 % formalin, we made also suspensions in:
B: saline with merthiolate 1 : 10000,
C: phosphate buffer pH 7 with formalin 0.5 %,
D: phosphate buffer pH 7 with merthiolate 1 : 10000.

The results of 2 experiments are summarized in table 1.

TABLE 1.

Agglutination test in two experiments with standard serum, of the bacteria in four antigens which were prepared in different ways.

	Agglutination titer	
	Exp. I	Exp. II
A. Antigen in saline + formalin 0.5 %	1 : 15360	1 : 15360
B. Antigen in saline + merthiolate	1 : 720	
C. Antigen in phosphate buffer + formalin 0.5 %	1 : 960	
D. Antigen in phosphate buffer + merthiolate	< 1 : 120	< 1 : 300

TABLE 2.

Agglutinin absorption test with two different prepared antigen suspensions.

	dilution	% agglutinin not absorbed
A. Antigen in saline + formalin	undiluted	5
	1/2	9.3
	1/4	33
	1/8	50
B. Antigen in phosphate buffer + merthiolate	undiluted	6
	1/2	10
	1/4	33
	1/8	75
Control serum		100

The agglutinogen content of the bacteria in antigen A was very high, that in the antigens B, C, and D was much lower. Apparently the agglutinogen is lost or destroyed by treatment with phosphate buffer or merthiolate. By means of the agglutinin absorption test we could show that the agglutinogen was not destroyed but had dissolved in the supernatant.

We mixed 0.25 ml from an $^1/_{30}$ dilution of an antiserum with 1 ml of antigen A in 4 concentrations, *viz.*, $^1/_1$, $^1/_2$, $^1/_4$, and $^1/_8$ and did the same with antigen D. After incubation during 1 hour at 37°C. and storing overnight in the refrigerator the antigen-serum mixtures were centrifuged. With the supernatants we did an agglutination test with a fresh *H. pertussis* suspension, in order to determine the

amounts of antibody (agglutinin) not absorbed by the antigens. Both antigens absorbed almost the same amount of agglutinins (table 2). Consequently the suspension A had the same agglutinogen content as the suspension D.

Discussion.

The agglutination test only gives the amount of agglutinogen on the bacteria, as the endpoint is estimated by a visible agglutination of the bacteria. In the agglutinin absorption test the amount of agglutinin of the serum (in excess) which is absorbed by the agglutinogen of the whole suspension is measured. As the agglutinin absorption test showed that the samples A and D contained the same amount of agglutinogen we must conclude that there was no destruction of agglutinogen in suspension D. The agglutinogen of the bacteria is dissolved in the buffer solution and therefore the bacteria showed a decreased agglutinogen content.

To prepare a stable antigen it is necessary to suspend the bacteria in saline with formalin in order to prevent the dissolution of the agglutinogen in the suspending medium. It may be that formalin in combination with saline plays a role as a fixative for the agglutinogen on the bacterial surface.

Summary.

The agglutinogen of *Hemophilus pertussis* is rather soluble in some suspending media but can be fixed on the bacterial surface with formaldehyde in saline.

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MODIFICATION OF THE HEREDITARY CHARACTER OF YEAST BY INGESTION OF CELL-FREE EXTRACTS [1)]

by

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(Received June 12, 1959).

I. Introduction.

Avery *et al.* reported in 1944 the transformation of a pneumococcus rough colony into the smooth colony by adding to the culture medium DNA originating from the smooth form. The pneumococcus strain "smooth" (S) has encapsulated cells, the "rough" colony type (R) has uncapsulated cells; the capsules of the transformed bacteria showed the same serologically type-specific polysaccharide.

The transformed bacterial cells continued to produce the ingested DNA, so the inheritable property of capsule synthesis was induced. Hotchkiss reported in 1955 that at least 30 distinct biochemical characters have been introduced *in vitro* by bacterial DNA into living cells of the homologous species. Recently Benoit *et al.* (1957) injected a DNA preparation derived from the Khaki Cambell duck into Peking ducklings and obtained a new race, which was different from the hybrid obtained in the normal way by crossing. Eight females and one male were obtained differing in the colour of the bill, the shape of the head, the carriage and the walk, the weight and the colour of the feathers. The following year a second generation was obtained, which showed the same characteristics, although the variation was greater than in normal hybridization experiments.

It seemed worthwhile to investigate the hereditary role of DNA in yeast. It is very difficult to cross brewing yeast on account of the poor sporulation and the bad viability of the spores, and on the rare

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1) A paper read at the E.B.C.-congress in Rome, 1959.

occasions when both spores germinate, they scarcely ever conjugate to form a hybrid (OPPENOORTH, 1956, 1959). It is nearly impossible to obtain hybrids by the spore-mating technique of WINGE, so the possibility of obtaining "hybrids" by the use of DNA preparations would offer a hopeful line of advance. It might be expected that, if it is possible to transform a yeast with DNA originating from another yeast, hybridisation by spore-mating of the transformed yeast with the original strain would be more successful.

II. METHODS AND MATERIALS.

1) Extraction.

In the preliminary experiments a modification of GAJDUSEK's method (1950) was used. Although at least 10 l yeast was used no visible precipitate was obtained. The preparations were biologically active but gave no chemical or physical reaction, so it must be inferred that the biological test is many times more sensitive than chemical or physical analysis.

A modified extraction according to GAJDUSEK was used: To 1 l pitching yeast 165 ml M NaCl and 10 g NaN_3 were added. During about half an hour this suspension was stirred on a water-bath at 50° – 60°C. till nearly all the cells were stained by Rhodamine B. The suspension was cooled rapidly to 4°C. and this temperature was maintained during the rest of the procedure. The yeast was centrifuged down at 3000 r.p.m., resuspended in 1 l distilled water and kept in the refrigerator overnight. Next day the yeast was centrifuged down, resuspended in 1 l distilled water and after shaking 4 hours the suspension was centrifuged and the two hazy supernatants were collected.

The combined solution was cleared by centrifuging at 30,000 g. The clear solution was decanted and NaCl was added to a final concentration of 0.14 M NaCl. After stirring a haze was formed. The precipitate was collected by centrifuging at high speed (30,000 g), dissolved in 100 ml distilled water and after stirring for half an hour to dissolve as much as possible the solution was cleared again by centrifuging at high speed. This procedure was repeated 2 or 3 times. After final precipitation 15 ml cooled wort was used to dissolve the precipitate and divided into 3 – 5 culture tubes. One tube was not inoculated and kept as a control for sterility, a second was transferred to Durham tubes to test for the presence of active enzymes while

the remainder were inoculated with the yeast under investigation.

The only micro-organisms which sometimes developed in the sterility controls were moulds. Active enzymes were never present.

The final precipitate from 1 l pitching yeast was too small to be visible or to be detected by chemical or physical methods but the wort containing the precipitate was active in the biological test. Increasing the yeast volume to 10 l did not give a visible precipitate.

Many extractions were performed and all the rejected fractions were tested for biological activity to find if DNA was being lost. Apparently only a part of the active substance is precipitated in 0.14 M NaCl which may explain conflicting reports in the literature. Following this discovery a new method was adopted. Although the controls never showed an infection with yeast, the method of GAJDUSEK did not give absolute certainty that no living cell was present in the precipitate. It was impossible to sterilise (20 minutes at 120°C.) or to pasteurise (one hour at 60°C.) or to filter the DNA solution through a Carlson EK sheet without complete loss of activity; therefore a precipitation with 70% ethanol was chosen.

The superiority of the new extraction method is shown by the fact that more DNA was obtained from 5 ml centrifuged yeast in this way than from 10 l pitching yeast by the former method.

The new extraction method.

A two days old culture of yeast in 1 l wort (cultivated at 25°C.) was centrifuged down (3000 r.p.m.) and washed thoroughly in distilled water. About 5 ml yeast was obtained. This was suspended in 10 ml of a saline citrate-phosphate buffer solution (Mc.Ilvain, diluted 1 : 7, pH 5.8, 0.14 M NaCl); the gut juice of one Helix pomatia L or H. (Cryptomphalus) asperea O.F. Muller 1774 was added and the mixture kept for 6 hours at 25°C.

After this treatment about 50% of the cells had lost their cell walls (EDDY, 1957), a number seemed intact while others had lysed. Some chloroform and 1% lauryl sulphate was added and the suspension stirred in a water-bath at 50° – 60°C. for about half an hour to kill all the cells. Then the suspension was cooled down to 5°C. and this temperature was maintained during the rest of the procedure.

The staining with Rhodamine B served as a control (OPPENOORTH, 1958). KAY *et al.* (1952, 1953) used dodecyl-sulphate as extracting and deproteinising agent, lauryl sulphate was equally effective. The chloroform was used to kill the cells (VENDRELY, 1956). The yeast

was then centrifuged down and resuspended in 0.14 M NaCl, the supernatant was kept. The sediment was washed several times with 0.14 M NaCl till about 100 ml decanted liquid was obtained. This solution was shaken with 5 ml activated charcoal, previously washed with a 1 M NaCl solution (ZAMENHOF and CHARGAFF, 1951) and cleared by centrifuging. The charcoal should retain the RNA and the depolymerised DNA. The decanted solution (about 100 ml) was shaken with 30 ml chloroform and 10 ml amyl alcohol (SEVAG *et al.*, 1938; BRAUN *et al.*, 1957; HOTCHKISS, 1957) to remove the proteins. The milky supernatant was decanted cautiously, the last drops were collected with a pipette. This milky supernatant could not be cleared by low speed centrifuging (3000 r.p.m.). The chloroform-amyl alcohol extraction was repeated several times, at least 4 times, till scarcely any gel was formed. The chloroform layer was rejected.

Two volumes of 96% ethanol were added and the precipitate collected by low speed centrifuging. The ethanol-water mixture was discarded. The precipitate was dissolved in 10 ml 0.14 M NaCl, cleared by centrifuging (3000 r.p.m.). The alcohol precipitation was repeated two times. Finally the precipitate was taken up in about 15 ml wort and divided into some culture tubes.

With the alcohol precipitation absolute sterility was ensured at this stage, so the solution in wort and subsequent addition to the sterile culture tubes had to be done with aseptic precautions, and the controls mentioned in the former extraction method were continued.

2) Controls.

To guard against the possibility that some living yeast cells might have survived the extraction procedure and had been carried over into the DNA wort, controlling experiments were run simultaneously with the transforming ones. The presence of active enzymes had also to be excluded.

One tube with wort containing DNA was not inoculated, it was kept with the inoculated tubes in the incubator and served as a control for sterility. Sometimes a mould developed but never yeast.

An equal volume of the wort containing DNA as was used to transform the yeast was divided into Durham tubes with the sugar broths under investigation (maltose, melibiose, raffinose, sucrose and galactose). This volume was at least 100 times larger than would normally reach the Durham tubes. After standing 30 days in the incubator at 25°C. none of the tubes showed any development of gas.

Thus it was ensured that the wort containing DNA carried with it neither living yeast cells nor active enzymes.

3) Analysis.

The most sensitive test for both DNA and RNA is to measure the absorption spectrum with a maximum at 2560 – 2580 Å and a minimum at 2300 Å. Unfortunately this spectrum is the same for both as absorption is due to the purine and pyrimidine bases. It is however a useful tool to judge the purity during the extraction and to estimate the concentration. When protein is present the maximum and minimum are shifted to the longer wave lengths.

Deoxyribonucleic acid (DNA).

DISCHE (1955) has summarised several methods of estimating DNA. The diphenyl amine reaction according to SEIBERT with the high peak at 5950 Å was preferred. Sometimes the results were compared with those obtained by STUMPF's modification of the cysteine reaction.

Ribonucleic acid (RNA).

The concentration of RNA was estimated by the orcinol reaction (λ 6700 Å), the phloroglucinol reaction (λ 6800 Å) and the cysteine reaction (λ 3900 Å), as reported by DISCHE (1955).

Protein.

The protein was estimated by the biuret reaction according to ROBINSON and HOGDEN (1940) and WEICHSELBAUM (1946) as referred to in Methods in Enzymology III (1957).

For these three substances calibration curves were made from materials obtained as follows:

The DNA as supplied by Schuchardt was prepared from herring sperm. The origin of the RNA (Quality I Schuchardt) was not stated but it was presumably prepared from yeast. Egg albumen was used as protein and gave the same curve as yeast protein obtained by the DNA extraction.

In control experiments, enzyme preparations were used to destroy the activity of the extract. To destroy DNA deoxyribonuclease (25% activity of cryst.) was used and for RNA ribonuclease (cryst., salt free, protease free) was used. Both were supplied by L. Light & Co. Ltd., England.

4) The yeasts.

a. **DNA Donators.**

Brewing yeast (*Saccharomyces carlsbergensis*) was delivered by two breweries as pitching yeast, one yeast was of the flocculating type and the other was powdery. Their fermenting abilities were equal and both showed poor sporulation and bad viability of the spores.

Before extraction the yeast was washed several times with distilled water. The cell wall was higly resistant to the gut juice of Helix.

Saccharomyces chevalieri was cultivated in sterilised brewery wort in one litre flasks in the incubator at 25°C. After 2-4 days growth the yeast was collected by low speed centrifuging and washed thoroughly with distilled water.

Saccharomyces chevalieri ferments raffinose 1/3, sucrose, galactose and glucose but not melibiose or maltose. The cell walls were readily digested by the gut juice of Helix.

b. **DNA Acceptors.**

R_7 is a yeast kindly supplied by Prof. WINGE and Dr. C. ROBERTS, labelled 303-9 and was descended from crossings between *Sacch. cerevisiae*, *Sacch. chevalieri* and *Sacch. italicus*. It can ferment galactose and glucose only. Its genotype is mm/meme/rr/gsgs. $K_{83}S_{58}$ belongs to the offspring of the hybrid K_{83} obtained by crossing a culture of brewing yeast with R_7 (OPPENOORTH, 1959). It is not able to ferment maltose, sucrose or raffinose but can ferment melibiose, galactose and glucose. Its genotype is mm/MeMe/rr/gsgs. One day old cultures in wort were used in the transforming experiments. Depending upon the inoculation it took one or two days before fermentation in the DNA-containing wort was strong. When this active stage was reached, sugar media in Durham tubes were inoculated. A positive reaction here indicated a transformation or pseudo-transformation and cells could be isolated from the sugar tubes by the micromanipulator.

5) Cultivation.

The yeast strains were kept on agar slants or wort tubes. As sporulating medium Fowel's agar was used. Only asci with 4 spores were isolated and dissected. The indication of the yeast strains was as follows: C_6 is a single cell isolation; C_6S_1 is a culture derived from

one isolated spore of C_6; $C_6S_1S_{11}$ means a culture derived from one isolated spore of C_6S_1.

The sugar broths were prepared with 10% yeast autolysate. Sucrose and raffinose broth contained 2% of sugar. For melibiose broth, 3% raffinose was inoculated with *Sacch. multisporus* (any yeast with 1/3 raffinose fermentation could be used). After final attenuation the culture was autolysed for 2 days at 45°C., filtered and sterilised and was then ready for use.

III. TRANSFORMATION AND PSEUDO-TRANSFORMATION.

1) Transformation.

a. Preliminary experiments.

A DNA-containing extract was made from a brewing yeast according to the method of GAJDUSEK (1950). This bottom yeast was able to ferment maltose, melibiose, raffinose, sucrose and galactose. This preparation was added to wort and the wort inoculated with R_7, which was not able to ferment the disaccharides and raffinose. Only after adaptation this yeast fermented galactose (slow fermenter).

After some days growth in the DNA wort a maltose Durham tube was inoculated and after two days a positive result was observed. From this maltose medium, cells were isolated with the micromanipulator. About 70% of the isolated cells were able to ferment not only maltose but also the other disaccharides and raffinose. In the maltose broth the percentage of the transformed cells had increased considerably. The transformed yeast retained the acquired ability during sub-culturing 20 times every second day into new wort tubes. Attempted de-adaptation by transferring ten times in glucose broth had no influence on the ability to ferment the disaccharides. When after this de-adaption period the yeast was transferred into maltose broth, the sugar was fermented as readily as before. The original yeast R_7 sporulated abundantly with enough asci with four spores to find them without difficulty. The brewing yeast on the contrary sporulated very poorly. Unfortunately the transformed yeast also sporulated poorly but some asci with two spores were found. Asci with three or four spores did not occur. In all 50 asci with two spores were dissected and the spores isolated. Only 15 cultures were obtained, all of which were able to ferment the disaccharides. That so few spores were viable was in agreement with the results of a compara-

TABLE 1.

The fermenting ability of isolated single cells from transformation experiments.

Fermentation of:		Melibiose	Raffinose	Sucrose	Galactose	Glucose			
Original strains:									
Saccharomyces chevalieri	W 332	—	$+^1/_3$	+	+sl	+	donator		
	$K_{83}S_{58}$	+	—	—	+sl	+	acceptor		
	R_7	—	—	—	+sl	+	acceptor		
							Genotype		
							Melibiose	Raffinose	Sucrose
Isolated cells of $K_{83}S_{58}$:	C_1	+	+	+	+	+	heteroz.	heteroz.	hom.d.
	C_2	—	—	—	+	+			
	C_3	+	+	+	+	+			
	C_4	+	+	+	+	+	heteroz.	heteroz.	hom.d.
	C_5	—	—	—	+	+			
	C_6	—	—	+	+	+	hom.r.	hom.r.	hom.d.
	C_7	+	—	+	+	+			
	C_8	+	+	+	+	+			
	C_{14}	+	+	+	+	+			
	C_{15}	—	+	+	+	+			
	C_{16}	—	—	+	+	+			
	C_{17}	+	+	+	+	+			
	C_{18}	+	+	+	+	+	heteroz.	heteroz.	heteroz.
	C_{19}	—	—	+	+	+	hom.r.	hom.r.	hom.d.
Isolated cells of R_7:	C_{21}		+	—					
	C_{25}		+	—					
	C_{28}		+	—					
	C_{31}		+	+					
	C_{33}		+	+					

hom.d. = homozygote dominant.
hom.r. = homozygote recessive.

Not all the isolated cells are transformed.
The transformed cells show all kinds of combinations of the genes.
It gives the impression of the segregation in a F_2.

ble crossing of a brewing yeast obtained in the normal way by crossing two spores. The asci with four spores obtained from that hybrid gave one or at most two viable spores. The others did not germinate or stopped growth in an early stage. It is obvious that a lethal factor or a combination of factors is interfering. It may be that only spores which possess the fermenting capacity are viable. WINGE and ROBERTS (1957) reported a similar result and suggested a vital gene (V) linked to the gene for sucrose fermentation.

b. Later experiments.

To localise the ingested DNA with the dominant hereditary characters, hybrids of the transformed yeast and the original recessive form had to be made. If the ingested DNA was used exclusively to build up the newly formed chromosomes it would be localised in the nucleus. In that case a hybrid obtained by orthodox crossing would give a segregation in fermenters and non-fermenters. When the ingested DNA remained in the cytoplasm no mendelian segregation would be obtained.

On account of the poor spore formation and bad viability of the spores, instead of brewing yeast, *Sacch.chevalieri* was used as DNA donator. As acceptor R_7 and $K_{83}S_{58}$ was used. The transformation succeeded and by reason of the better sporulation and viability of the spores the experiments were continued with the progeny of $K_{83}S_{58}$.

With the micromanipulator cells were isolated from the sugar broth in the positive Durham tubes and the resulting colonies tested. In contrast to the first experiments all kinds of combinations of fermenting characters were found as shown in table 1.

From a culture of $K_{83}S_{58}$ grown in wort containing DNA some cells could ferment melibiose, raffinose and sucrose, some sucrose only and so on. Often the capacity to ferment melibiose was absent. The transformation rate of R_7 was less, and from the 50 isolated cells only a few were transformed. It may be that the activity of the extracts used in these experiments varied considerably. The yield of the extraction was not always the same and sometimes an extract did not cause a transformation.

2) Pseudo-transformation.

A phenomenon which gave rise to some difficulty at first might be classed as pseudo-transformation and described as an induced tem-

porary ability to ferment sugar which is lost after some transfers into the same sugar broth. An example of this is set out in table 2.

TABLE 2.

Pseudo-transformation.

An extract of *Saccharomyces chevalieri* is ingested by $K_{83}S_{58}$. The wort containing the extract gives no positive result in the sucrose Durham tube. The first transfer of the "transformed" yeast into sucrose broth gave a positive reaction. After some transfers from sucrose broth into sucrose broth the reaction diminished and disappeared completely.

	sucrose Durham tube
Sacch. chevalieri	positive after 1 day
$K_{83}S_{58}$	negative during 30 days
Control, wort with extract	negative during 30 days
"transformed" $K_{83}S_{58}$	
first transfer	positive after 1 day
2nd ,,	positive after 3 days
3rd ,,	positive (½) after 7 days
4th ,,	one bubble after 14 days
5th ,,	negative during 30 days

When "½" or "bubble" is reported, no increase of the gas volume was observed after further incubation.

$K_{83}S_{58}$ is fed with an extract from *Sacch.chevalieri*. Neither $K_{83}S_{58}$ nor the wort containing the extract gave a positive reaction in the sucrose Durham tube. The first transfer of $K_{83}S_{58}$ from the induction medium to the sucrose Durham tube gave a positive reaction after one day culturing at 25°C. The second transfer from the first Durham sucrose tube into a second one showed a positive reaction after 3 days, the third transfer showed after 7 days a positive reaction but the inverted tube was only half filled by the gas before fermentation ceased entirely. The fourth transfer showed only one bubble after 14 days and the fifth transfer gave no positive reaction.

So here we have a de-adaptation taking place in the sugar broth. The fermenting ability for the sugar was lost during cultivation in that sugar. To be certain of a true transformation it proved necessary to transfer the yeast several times in the same sugar broth and to note the time for a positive reaction in each successive transfer. In the case of true transformation the time interval decreases.

IV. Genetics of the isolated single cells.

To get information about the genotype of the isolated single trans-

formed cells the spores had to be isolated and tested for fermenting ability. The characteristics of the isolated single cells are summarised in table 1. The pedigree of the tested cells is summarised in the following tables.

TABLE 3.

The fermenting ability of the isolated single spores of C_1 and the adaptation time.

		Melibiose	Raffinose	Sucrose		
	C_1	+	+	+		
Ascus 1	S_1	—	—	—	melibiose fermentation	8+ : 8—
	S_2	+	+	+	1 ascus	3+ : 1—
	S_3	—	—	+	2 asci	2+ : 2—
	S_4	—	—	+	1 ascus	1+ : 3—
Ascus 2	S_5	+	—	+	raffinose fermentation	7+ : 9—
	S_6	—	—	—	1 ascus	3+ : 1—
	S_7	+	+	+	1 ascus	2+ : 2—
	S_8	—	—	+	2 asci	1+ : 3—
Ascus 3	S_9	+	+	—	sucrose fermentation	10+ : 6—
	S_{10}	+	—	—	2 asci	3+ : 1—
	S_{11}	+	+	+	2 asci	2+ : 2—
	S_{12}	—	—	+		
Ascus 4	S_{13}	+	+	—		
	S_{14}	—	—	+		
	S_{15}	—	—	+		
	S_{16}	+	+	—		

Adaptation time of some cultures.

raffinose	1st transfer	2nd transfer
C_1S_2	+ 14 days	+ 3 days
C_1S_{11}	+ 15 days	+ 4 days
sucrose		
C_1S_5	½ + 15 days	½ + 6 days
C_1S_{14}	bubble 7 days	½ + 7 days

When "bubble" or "½" is reported, no increase of the gas volume could be noticed after further incubation.

C_1 is heterozygote for melibiose and raffinose and presumably homozygote dominant for sucrose.

C_1 (table 3).

C_1 could ferment melibiose, raffinose and sucrose. Six asci with 4 spores were dissected and of 4 asci all the spores developed. Three spores from 2 asci failed to germinate. The characteristics of the resulting colonies are set out in table 3.

The original $K_{83}S_{58}$ was homozygote: dominant for melibiose-fermentation, recessive for raffinose and sucrose. C_1 on the contrary is heterozygote for melibiose and raffinose but the genotype of sucrose fermentation is not definite. Presumably it is homozygote dominant and it is supposed that some spores have lost the ability to ferment sucrose. In nearly all the experiments the sucrose fermentation gave difficulties. It turned out that the fermentation is of an adaptive type, as can be illustrated by the following:

raffinose:	C_1S_2	first transfer positive after 14 days
		second transfer positive after 3 days.
	C_1S_{11}	first tube positive after 15 days
		second tube positive after 4 days.
sucrose:	C_1S_4	first transfer after 15 days half
		second transfer after 6 days half.
	C_1S_{14}	first transfer after 7 days 1 bubble
		second transfer after 7 days half.

These experiments indicate that the gene for melibiose fermentation can be ousted from the chromosome by the assimilated DNA and it is likely that the gene for raffinose fermentation from *Sacch. chevalieri* is localised exclusively in the nucleus on account of the distinctly mendelian segregation. The behaviour of other isolated cells supports this view.

In the hybridisation experiments C_1S_2 and C_1S_{11} were used. To determine their genotype three asci with 4 spores were dissected from each of the two strains. All the spores germinated, developed into colonies and were tested. Table 4 and 5 and diagram 1 show the results.

In the pedigree of C_1S_2 three colonies lost the ability to ferment sucrose, of C_1S_{11} two spores did not ferment raffinose and three spores did not ferment sucrose. Although this means that C_1S_2 and C_1S_{11} were homozygous dominant for the three sugars account must be taken of the fact that the ability to ferment a sugar can be lost. Furthermore it is to be concluded that the assimilated substance must be able to multiply itself, otherwise it becomes so diluted that it fails to find expression in visible fermentation.

TABLE 4.

The fermenting ability of the isolated spores of C_1S_2.

		Melibiose	Raffinose	Sucrose
	C_1S_2	+	+	+
Ascus 1	S_1	+	+	—
	S_2	+	+	+
	S_3	+	+	+
	S_4	+	+	+
Ascus 2	S_5	+	+	+
	S_6	+	+	+
	S_7	+	+	+
	S_8	+	+	+
Ascus 3	S_9	+	+	+
	S_{10}	+	+	+
	S_{11}	+	+	—
	S_{12}	+	+	—

Three spores lost the ability to ferment sucrose.

C_1S_2 is homozygote dominant for melibiose, raffinose and sucrose.

TABLE 5.

The fermenting ability of the isolated spores of C_1S_{11}.

		Melibiose	Raffinose	Sucrose
	C_1S_{11}	+	+	+
Ascus 1	S_1	+	+	+
	S_2	+	+	+
	S_3	+	+	—
	S_4	+	+	+
Ascus 2	S_5	+	+	+
	S_6	+	+	—
	S_7	+	+	+
	S_8	+	—	+
Ascus 3	S_9	+	+	—
	S_{10}	+	+	+
	S_{11}	+	—	+
	S_{12}	+	+	+

Two spores lost the ability to ferment raffinose and 3 spores lost the sucrose fermentation.

C_1S_{11} is homozygote dominant for melibiose, raffinose and sucrose.

DIAGRAM 1.
Segregation of the fermenting ability of C_1.

		Fermentation of the progeny		
		Melibiose	Raffinose	Sucrose
S_5	F_4	4+ : 18—	4+ : 18—	22+ : 0—
S_2	F_3	12+ : 0—	12+ : 0—	9+ : 3—
C_1	F_2	8+ : 8—	7+ : 9—	10+ : 6—
S_{11}	F_3	12+ : 0—	10+ : 2—	9+ : 3—
S_4	F_4	15+ : 0—	9+ : 6—	3+ : 12—

In the progeny of $C_1S_2S_5$ seven cultures have lost the ability to ferment melibiose, although this character was not an induced one. The induced ability to ferment sucrose was retained throughout.

The results obtained with the progeny of $C_1S_{11}S_4$ are just the reverse.

C_4 (table 6).

C_4 could ferment melibiose, raffinose and sucrose. Eight asci with 4 spores were dissected, from 6 asci all the spores developed completely. From 2 asci three spores did not germinate and these two are not included. The results are summarised in table 6. The segregation for melibiose and raffinose is in all asci exactly 2+ : 2—. For sucrose there is a shortage of 7 fermenters indicating homozygoty for sucrose. Here again a linkage between melibiose and the newly acquired raffinose fermentation can be observed.

C_6 (table 7, diagram 2).

C_6 could ferment sucrose but not melibiose and raffinose. Three asci were dissected and all the spores developed. None of the derived colonies could ferment melibiose or raffinose, of the 12 colonies 8 could ferment sucrose and 4 had lost the ability. So C_6 must be homozygote recessive for melibiose and raffinose fermentation and presumably homozygote dominant for sucrose. The original strain was homozygote dominant for melibiose. Of two colonies deriving

from single spores of C_6, C_6S_1 and C_6S_2 spores were isolated. These two could ferment sucrose only.

TABLE 6.

The fermenting ability of isolated single spores of C_4.

		Melibiose	Raffinose	Sucrose		
	C_4	+	+	+		
Ascus 1	S_1	+	+	+	melibiose fermentation	12+ : 12—
	S_2	—	—	+	6 asci	2+ : 2—
	S_3	—	—	—		
	S_4	+	+	+		
Ascus 2	S_5	+	+	+	raffinose fermentation	12+ : 12—
	S_6	+	+	—	6 asci	2+ : 2—
	S_7	—	—	+		
	S_8	—	—	+		
Ascus 3	S_9	+	+	+	sucrose fermentation	17+ : 7—
	S_{10}	—	—	+	1 ascus	4+ : 0—
	S_{11}	+	+	+	3 asci	3+ : 1—
	S_{12}	—	—	+	2 asci	2+ : 2—
Ascus 4	S_{13}	—	—	—		
	S_{14}	+	+	+		
	S_{15}	+	+	—		
	S_{16}	—	—	+		
Ascus 5	S_{17}	+	+	—		
	S_{18}	+	+	+		
	S_{19}	—	—	+		
	S_{20}	—	—	+		
Ascus 6	S_{25}	—	—	—		
	S_{26}	+	+	+		
	S_{27}	—	—	—		
	S_{28}	+	+	+		

C_4 is heterozygote for melibiose and raffinose and presumably homozygote dominant for sucrose.

C_6S_1 (table 8).

Three asci were dissected, all the 12 spores developed. None of the derived colonies could ferment melibiose or raffinose, all except two could ferment sucrose (table 8).

C_6S_{11} was spored and eight asci were dissected, the spores of 6 asci developed completely, the other two asci were rejected (table

TABLE 7.

The fermenting ability of isolated single spores of C_6.

		Meli-biose	Raffi-nose	Suc-rose	
	C_6	—	—	+	
Ascus 1	S_1	—	—	+	Melibiose: homozygote recessive
	S_2	—	—	+	Raffinose: homozygote recessive
	S_3	—	—	+	
	S_4	—	—	—	
Ascus 2	S_5		—	—	Sucrose: 8+ : 4—
	S_6		—	+	2 asci 3+ : 1—
	S_7		—	+	1 ascus 2+ : 2—
	S_8		—	+	
Ascus 3	S_9		—	+	
	S_{10}		—	—	
	S_{11}		—	—	
	S_{12}		—	+	

Four spores lost the ability to ferment sucrose.
C_6 is presumably homozygote dominant for sucrose.

DIAGRAM 2.

Segregation of the fermenting ability of C_6.

		Fermentation of the progeny.		
		Melibiose	Raffinose	Sucrose
S_{11}	F_4	0+ : 24—	0+ : 24—	8+ : 16—
S_1	F_3	0+ : 12—	0+ : 12—	10+ : 2—
C_6	F_2	0+ : 12—	0+ : 12—	8+ : 4—
S_2	F_3	0+ : 12—	0+ : 12—	5+ : 7—

9). Of the 24 cultures only eight could ferment sucrose. After 32 days the other 16 had grown but did not ferment the sugar and chemical analysis showed this was still present. In this as in other instances the newly acquired ability was lost by many spores which seriously interferes with the genetic analysis. Probably there are restrictions to the automultiplication of the foreign DNA or the

TABLE 8.

The fermenting ability of isolated single spores of C_6S_1.

		Meli-biose	Raffi-nose	Suc-rose	
	C_6S_1	—	—	+	
Ascus 1	S_1	—	—	+	Melibiose: homozygote recessive
	S_2	—	—	+	Raffinose: homozygote recessive
	S_3	—	—	+	
	S_4	—	—	+	
Ascus 2	S_5			—	Sucrose: 10+ : 2—
	S_6			+	2 asci 4+ : 0—
	S_7			—	1 ascus 2+ : 2—
	S_8			—	Presumable homozygote dominant
Ascus 3	S_9			+	
	S_{10}			+	
	S_{11}			+	
	S_{12}			+	

organisation of the cell hampers its manifestation. In the latter case there will be a difference between the genotype and the phenotype.

It cannot be a question of dilution because each colony of millions of cells originates from one spore and obviously there must have been some reproduction of the ingested DNA otherwise the acquired ability would very soon diminish to vanishing point.

C_6S_2 (table 10).

Three asci were dissected, all the 12 spores developed. No fermentation of melibiose or raffinose was observed. Five colonies could ferment sucrose, 7 colonies had lost the ability.

In diagram 2 the segregation experiments of C_6 are surveyed. In the progeny of $C_6S_1S_{11}$, 16 of the 24 spores have lost the ability to ferment sucrose. It should be emphasised that loss of fermenting ability is never observed during vegetative propagation but only after generative propagation. This indicates that the reproduction of DNA sometimes fails. This might be called mutation but the author hesitates to use this term without being able to explain its mechanism.

C_8.

C_8 had retained the ability to ferment melibiose and had acquired

TABLE 9.

The sucrose fermenting of the isolated single spores of $C_6S_1S_{11}$.

		Sucrose	Chemical analysis of sucrose
$C_6S_1S_{11}$		+	
Ascus 1	S_1	+	
	S_2	—	unaffected
	S_3	—	unaffected
	S_4	+	
Ascus 2	S_5	+	
	S_6	—	unaffected
	S_7	—	unaffected
	S_8	—	unaffected
Ascus 3	S_9	+	
	S_{10}	—	unaffected
	S_{11}	—	unaffected
	S_{12}	—	unaffected
Ascus 4	S_{13}	—	unaffected
	S_{14}	+	
	S_{15}	+	
	S_{16}	—	unaffected
Ascus 5	S_{17}	+	
	S_{18}	—	unaffected
	S_{19}	—	unaffected
	S_{20}	—	unaffected
Ascus 6	S_{21}	—	unaffected
	S_{22}	+	
	S_{23}	—	unaffected
	S_{24}	—	unaffected

	sucrose fermentation
2 asci	2+ : 2—
4 asci	1+ : 3—

From each ascus 2 spores germinated rapidly and 2 spores slowly. 32 days after inoculation tests were made for sucrose when no positive reaction was observed. In all cases the sucrose was unaffected.

On account of the descent $C_6S_1S_{11}$ had to be:
homozygote recessive for melibiose and raffinose
homozygote dominant for sucrose.

the ability to ferment raffinose and sucrose. 24 asci were dissected and 17 asci developed completely. The other 7 were rejected. The results of the test on melibiose and raffinose were:

13 asci	2 fermenters :	2 non-fermenters
2 asci	1 ,, :	3 ,, ,,
2 asci	0 ,, :	4 ,, ,,

TABLE 10.

The fermenting ability of isolated single spores of C_6S_2.

		Melibiose	Raffinose	Sucrose	
	C_6S_2	—	—	+	
Ascus 1	S_1	—	—	+	Melibiose: homozygote recessive
	S_2	—	—	—	Raffinose: homozygote recessive
	S_3	—	—	+	
	S_4	—	—	+	
Ascus 2	S_5			—	Sucrose: 5+ : 7—
	S_6			+	1 ascus 3+ : 1—
	S_7			—	2 asci 1+ : 3—
	S_8			—	
Ascus 3	S_9			—	
	S_{10}			—	
	S_{11}			+	
	S_{12}			—	

The fermentation of raffinose started after about 25 days by which time it could be seen that the yeast was fully grown. After transferring the yeast from a positive raffinose tube to a second one, the positive result was obtained after about 4 days and a normal speed was attained when the yeast was fully adapted. The raffinose fermentation was always 1/3 although melibiose could be fermented – an inexplicable combination of results which calls for further investigation. The ability to ferment melibiose (old) and raffinose (new) was always linked.

C_8 was heterozygote for melibiose and raffinose (as C_1). The fermentation of sucrose was not investigated.

C_{16}.

C_{16} could ferment sucrose, but not melibiose and raffinose. 12 asci were dissected, 5 asci developed completely, the others were rejected. Of 4 asci all the spores could ferment sucrose, of the fifth ascus 3 spores could ferment, but one spore fermented nothing after 40 days.

None of the spores could ferment melibiose or raffinose. Thus the ability to ferment melibiose was lost and the ability to ferment sucrose (homozygous) was acquired.

C_{18} (table 11).

The single cell isolation C_{18} was able to ferment the sugars melibiose, raffinose and sucrose.

Three asci were dissected and all the spores developed. The fermenting abilities of the spores were tested and the results are summarised in table 11.

TABLE 11.

The fermenting ability of isolated single spores of C_{18}.

		Meli-biose	Raffi-nose	Suc-rose	
	C_{18}	+	+	+	
Ascus	$C_{18}S_1$	—	—	—	Melibiose
1	S_2	+	+	—	3 asci 2+ : 2—
	S_3	+	+	+	
	S_4	—	—	+	Raffinose
Ascus	S_5	+	—	—	2 asci 2+ : 2—
2	S_6	—	—	+	1 ascus 0+ : 4—
	S_7	—	—	+	
	S_8	+	—	—	Sucrose
Ascus	S_9	—	—	—	3 asci 2+ : 2—
3	S_{10}	+	+	—	
	S_{11}	+	+	+	
	S_{12}	—	—	+	

In ascus Nr. 2 the raffinose fermenting ability is lost.
C_{18} is heterozygous for melibiose, raffinose and sucrose.

It turned out that C_{18} was heterozygous for all the three sugars, even the sucrose fermentation was quite definite. Only in ascus number 2 the spores had lost the ability to ferment raffinose.

C_{19} (table 12).

The single cell isolation C_{19} was able to ferment sucrose but not melibiose or raffinose.

Six asci were dissected and the spores of 4 developed completely. The others were rejected. None of the spores could ferment melibiose or raffinose (table 12). All the spores except one could ferment sucrose. The genotype of C_{19} was homozygote recessive for melibiose and raffinose and homozygous dominant for sucrose.

V. Hybridisation experiments.

Another method of localising the ingested substance consists in hybridising transformed cells together or transformed cells with the original recessive yeast.

TABLE 12.

The fermenting ability of isolated single spores of C_{19}.

		Melibiose	Raffinose	Sucrose
	C_{19}	—	—	+
Ascus 1	$C_{19}S_{53}$	—	—	+
	S_{54}	—	—	+
	S_{55}	—	—	—
	S_{56}	—	—	+
Ascus 2	S_{57}	—	—	+
	S_{58}	—	—	—
	S_{59}	—	—	+
	S_{60}	—	—	+
Ascus 3	S_{65}	—	—	+
	S_{66}	—	—	+
	S_{67}	—	—	+
	S_{68}	—	—	+
Ascus 4	S_{69}	—	—	+
	S_{70}	—	—	+
	S_{71}	—	—	+
	S_{72}	—	—	+

C_{19} is homozygote recessive for melibiose and raffinose, homozygote dominant for sucrose.

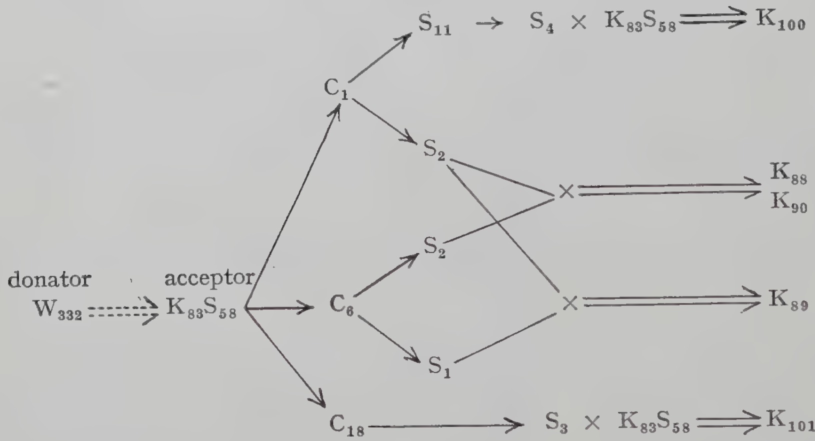
The spore mating technique of WINGE was used. Only when indubitable conjugation was observed and a zygote formed was the hybrid accepted. It was noticed that hybrids were formed much more easily in these experiments than in crossings between the original strains. When the hybrid had developed into a micro-colony in the droplet on the coverslip it was transferred to a wort tube. Then the fermenting characters were tested. If the expected characters were present, single cell isolations were made and tested again to be quite sure about the purity and the success of the crossing.

It was often apparent that the first wort tube contained a mixture of cells of the hybrid and of one or both of the ancestors. Sometimes no hybrid cells were found, although a zygote had been observed. Presumably the nuclei had not fused. Therefore only single cell isolations of a zygote were used in the further experiments. In a previous paper (OPPENOORTH, 1959) it was demonstrated that cell fusion could occur without the fusion of the nuclei. In diagram 3 a scheme of the hybridisation experiments is given. K_{88}, K_{89} and K_{90} were ob-

tained by crossing transformed cells, K_{100} and K_{101} originated by crossing transformed cells with the original yeast.

DIAGRAM 3.

Hybridization experiments with transformed cells.



K_{88} (table 13).

K_{88} was obtained by crossing C_1S_2 with C_6S_2. Originally it was thought that C_1S_2 could not ferment sucrose. After a long adaptation time, however, it could do so. C_1 still did not ferment sucrose after 20 days, C_1S_2 was positive after 12 days and the second transfer after 3 days. K_{88} had to be homozygous for sucrose fermentation; in the F_2, however, there was a segregation 45+ : 51—.

The segregation within the completely developed asci was quite different. Six of the 15 asci showed a 4+ : 0— segregation.

There is a possibility that two linked factors are involved, but it seems more probable that the ability has been lost. The melibiose fermentation looks like a normal mendelian segregation for one factor, apart from one ascus with three fermenters accompanied by one spore which had lost this ability. The fermentation of raffinose looks the same taking into account the ease with which the newly acquired abilities can be lost. The linkage between melibiose and raffinose is still maintained. No linkage between melibiose and sucrose or sucrose and vital gene could be noticed.

K_{89} (table 14).

K_{89} originated from a cross of C_1S_2 with C_6S_1 and could ferment

TABLE 13.

K_{88} ($=C_1S_2 \times C_6S_2$).

	Melibiose	Raffinose	Sucrose
C_1S_2	+	+	+
C_6S_2	—	—	+
K_{88}	+	+	+

29 asci isolated
15 asci: all 4 spores developed
14 asci: 20 spores did not germinate (17%)
96 spores gave cultures (83%)

Segregation of all spores (96).

Segregation	+	—
Theoretical	48	48
Melibiose	43	53
Raffinose	37	59
Sucrose	45	51

Segregation in complete asci (15).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	—	—	6
3+ : 1—	1	—	1
2+ : 2—	13	11	7
1+ : 3—	—	2	1
0+ : 4—	1	2	—

Linkage between melibiose (genuine) and raffinose (induced):
Melibiose+ & Raffinose+ 24 times
Melibiose+ & Raffinose— 5 times
Melibiose— & Raffinose+ none
Melibiose— & Raffinose— 31 times
No linkage between: melibiose and sucrose, sucrose and vital gene.

TABLE 14.

K_{89} (= C_1S_2 × C_6S_1).

	Melibiose	Raffinose	Sucrose
C_1S_2	+	+	+
× C_6S_1	—	—	+
K_{89}	+	+	+

30 asci isolated
18 asci completely developed
12 asci: 18 spores did not germinate (15%)
102 spores gave cultures (85%)

Segregation of all spores (102).

Segregation	+	—
Theoretical	51	51
Melibiose	47	55
Raffinose	37	65
Sucrose	74	28

Segregation in complete asci (18).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	—	—	6
3+ : 1—	—	—	9
2+ : 2—	15	11	2
1+ : 3—	—	3	1
0+ : 4—	3	4	—

Linkage between melibiose (genuine) and raffinose (induced):
Melibiose+ & Raffinose+ 25 times
Melibiose+ & Raffinose— 5 times
Melibiose— & Raffinose+ none
Melibiose— & Raffinose— 42 times
No linkage between: melibiose and sucrose, sucrose and vital gene.

TABLE 15.

K_{90} (=C_1S_2 × C_6S_2).

	Melibiose	Raffinose	Sucrose
C_1S_2	+	+	+
× C_6S_2	—	—	+
K_{90}	+	+	+

28 asci isolated
17 asci completely developed
11 asci: 18 spores did not germinate (16%)
94 spores gave cultures (84%)

Segregation of all spores (94).

Segregation	+	—
Theoretical	47	47
Melibiose	47	47
Raffinose	39	55
Sucrose	54	40

Segregation in complete asci (17).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	1*)	1*)	3
3+ : 1—	—	—	6
2+ : 2—	15	11	5
1+ : 3—	—	4	3
0+ : 4—	1	1	—

*) the same ascus

Linkage between melibiose and raffinose:
Melibiose+ & Raffinose+ 30 times
Melibiose+ & Raffinose— 4 times
Melibiose— & Raffinose+ none
Melibiose— & Raffinose— 34 times
No linkage between: melibiose and sucrose, sucrose and vital gene.

TABLE 16.

$K_{88} + K_{89} + K_{90}$.

	Asci		Spores		Segregation (of all spores)		
	isolated	completely developed	dead	developed	Melibiose + —	Raffinose + —	Sucrose + —
K_{88}	29	15	20 (17%)	96 (83%)	43 : 53	37 : 59	45 : 51
K_{89}	30	18	18 (15%)	102 (85%)	47 : 55	37 : 65	74 : 28
K_{90}	28	17	18 (16%)	94 (84%)	47 : 47	39 : 55	54 : 40
Total	87	50	56 (16%)	292 (84%)	137 : 155	113 : 179	173 : 119
				theoretical	146 : 146	146 : 146	292 : 0

Segregation in complete asci (50).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	1	1	15
3+ : 1—	1	—	16
2+ : 2—	43	33	14
1+ : 3—	—	9	5
0+ : 4—	5	7	—

Linkage between melibiose (genuine) and raffinose (induced):

Melibiose+	& Raffinose+	79 times
Melibiose+	& Raffinose—	14 times
Melibiose—	& Raffinose+	0 times
Melibiose—	& Raffinose—	107 times

the three sugars as expected. The segregation of melibiose and raffinose pointed to a normal mendelian segregation. The results obtained on the fermentation of sucrose showed a smaller loss of fermenters.

K_{90} (table 15).

The origin of K_{90} was the same as that of K_{88}. The results of the investigations of the F_2 were similar. In one completely developed ascus all the 4 spores could ferment both melibiose and raffinose. The fermentation of sucrose again showed the typical deviating segregation. There is no evidence of a linkage if we assume that two factors are responsible.

$K_{88} + K_{89} + K_{90}$ (table 16).

These three hybrids should be identical and this was fully confirmed by the F_2, so the results can be summarised together. In total 87 asci were dissected and from 50 asci all the spores developed. The viability of the spores was quite good (84%). The segregation in the fully developed asci pointed to a fairly normal mendelian segregation for melibiose and raffinose fermentation, induced by one factor each. The linkage between melibiose and raffinose is strong, there were no spores which could ferment raffinose and not melibiose.

The sucrose fermentation was less conclusive. It was assumed that the hybrids were homozygous dominant, but that in many spores the acquired ability was lost. It is not yet clear why the induced ability to ferment sucrose was lost so much more easily than that of raffinose.

K_{100} (table 17).

K_{100} was obtained by crossing back C_1S_{11} with the original yeast $K_{83}S_{58}$.

The sporulation of the hybrid and the viability of the spores was quite good. Unfortunately the spore of C_1S_{11} which was used to build up the zygote, must have lost the ablity to ferment melibiose. This had not previously been noticed in the progeny of C_1S_{11} or $C_1S_{11}S_4$ although the possibility was shown in the progeny of $C_1S_2S_5$ (diagram 1). The segregation of melibiose and raffinose fermentation looks like a normal mendelian one.

A linkage between melibiose and raffinose appeared. The segregation of sucrose fermentation, however, is again mysterious. The ratio of all the spores (45+ : 56—) should indicate one set of factors with the familiar overbalance of recessives for the induced characters. But judging from the ratio encountered in the completely developed asci, it is possible that the hybrid could have been homozygote for sucrose. In that case $K_{83}S_{58}$ had mutated from sucrose negative to sucrose positive.

Unfortunately this backcross looses its convincing character through these two aberrations.

K_{101} (table 18).

K_{101} was obtained by crossing back $C_{18}S_3$ with the original yeast $K_{83}S_{58}$.

TABLE 17.

K_{100} (= $K_{83}S_{58}$ × C_1S_{11}).

	Melibiose	Raffinose	Sucrose
$K_{83}S_{58}$	+	—	—
× C_1S_{11}	+	+	+
K_{100}	+	+	+

29 asci isolated
19 asci: all 4 spores developed
10 asci: 14 spores did not germinate (12%)
182 spores gave cultures (88%)

Segregation of all spores (102).

Segregation	+	—
Theoretical	51	51
Melibiose	50	52
Raffinose	30	72
Sucrose	46	56

Segregation in complete asci (19).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	—	—	3
3+ : 1—	2	1	3
2+ : 2—	16	7	4
1+ : 3—	1	8	3
0+ : 4—	—	3	6

Linkage between melibiose and raffinose:
Melibiose+ & Raffinose+ 23 times
Melibiose+ & Raffinose— 16 times
Melibiose— & Raffinose+ 2 times
Melibiose— & Raffinose— 35 times
No linkage between: melibiose and sucrose, sucrose and vital gene.

TABLE 18.

K_{101} (=$K_{83}S_{58}$ × $C_{18}S_3$).

	Melibiose	Raffinose	Sucrose
$K_{83}S_{58}$	+	—	—
× $C_{18}S_3$	+	+	+
K_{101}	+	+	+

28 asci isolated
17 asci: all 4 spores developed
11 asci: 14 spores did not germinate (12.5%)
98 spores gave cultures (87.5%).

Segregation of all spores (98).

Segregation	+	—
Theoretical	49	49
Melibiose	41	57
Raffinose	35	63
Sucrose	47	51

Segregation in complete asci (17).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	—	—	—
3+ : 1—	—	—	2
2+ : 2—	13	8	7
1+ : 3—	2	7	8
0+ : 4—	2	2	—

Linkage between melibiose and raffinose:
Melibiose+ & Raffinose+ 21 times
Melibiose+ & Raffinose— 7 times
Melibiose— & Raffinose+ 2 times
Melibiose— & Raffinose— 38 times
No linkage between: melibiose and sucrose, sucrose and vital gene.

TABLE 19.

$K_{100} + K_{101}$.

	Asci		Spores		Segregation (of all spores)		
	isolated	completely developed	dead	developed	Melibiose + : —	Raffinose + : —	Sucrose + : —
K_{100}	29	19	14 (12%)	102 (88%)	50 : 52	30 : 72	46 : 56
K_{101}	28	17	14 (12.5%)	98 (87.5%)	41 : 57	35 : 63	47 : 51
Total	57	36	28 (12.3%)	200 (87.7%)	91 : 109	65 : 135	93 : 107
				theoretical	100 : 100	100 : 100	100 : 100

Segregation in complete asci (36).

Ratio	Number of asci		
	Melibiose	Raffinose	Sucrose
4+ : 0—	—	—	3
3+ : 1—	2	1	5
2+ : 2—	29	15	11
1+ : 3—	3	15	11
0+ : 4—	2	5	6

Linkage between melibiose and raffinose:

Melibiose+	& Raffinose+	44 times
Melibiose+	& Raffinose—	23 times
Melibiose—	& Raffinose+	4 times
Melibiose—	& Raffinose—	73 times

The sporulation of the hybrid and the viability of the spores was equal to that of K_{100}. Here again the segregation of melibiose fermentation pointed to a heterozygoty for melibiose and here too $C_{18}S_3$ must have lost the ability to ferment melibiose. The segregation for raffinose and sucrose did not deviate seriously from the 2:2 ratio. Melibiose and raffinose were again linked.

$K_{100} + K_{101}$ (table 19).

Since the hybrids K_{100} and K_{101} must be the same on account of the genotype of the parents, the results are combined in table 19. Only the fermentation of sucrose in K_{100} was erratic.

Comparing these results with those obtained by crossing transformed cells together (as in table 16) no differences could be observed.

The sporulation of the hybrids and the viability of the spores were the same, the segregation of the fermentation of melibiose and raffinose both pointed to a fairly normal mendelian one. Only the fermentation of sucrose was mysterious. It must be assumed that the acquired fermenting abilities can be lost rather easily. The change of one sucrose negative gene into a sucrose positive one in the hybrid K_{100} can only be described as a mutation, but as already pointed out the author has an objection to this term in these investigations because it brings in a word without offering any explanation.

VI. The role of DNA and RNA.

In chapter II the transformation and pseudo-transformation were dealt with but the inducing agents were not specifically defined. In the literature examples of similar "transformation" can be found but the "pseudo-transformation" was hitherto unknown.

In the course of these studies a working hypothesis was set up in which it was assumed that the inheritable transformation was due to the ingested DNA. As the extracts were usually contaminated with RNA and protein, one of these other substances might induce the pseudo-transformation which faded out, presumably by dilution and was not heritable. One possibility was that the extracts contained enzyme precursors of proteinous nature. The extracts were not active in the fermentation tests but the living cells could build up enzymes from the ingested precursors and start fermenting. As soon as the supply of precursor would be exhausted, the ability to ferment would decrease with increasing number of cells.

The other possibility was that RNA was taken up by the cell and once arrived within the cytoplasm would be able to synthesise enzymes.

By cell division the ability would fade out through dilution because RNA is not able to multiply itself like DNA. If this last possibility could be confirmed it would indicate the function of RNA in the cell.

To test this possibility, extracts were made from *Sacch.chevalieri*. Besides the normal controls parts of the extracts were treated with the enzymes deoxyribonuclease (DNAase) which destroyed the DNA, ribonuclease (RNAase) which destroyed the RNA, and papain with pepsin which destroyed the proteins. In table 20 the results of one experiment are shown.

The transformation for sucrose fermentation was studied. The

TABLE 20.

Transformation of K_{83} S_{58} with DNA of *Sacch. chevalieri.*

Treatment	Durham tube sucrose broth			
	1st	2nd	3rd	4th
control	+ 2d	+ 4d	1/2 + 13d	1/2 + 13d
DNAase	+ 4d	b. 21d	—	—
RNAase	+ 3d	1/3 + 21d	1/3 + 13d	+ 6d
proteolytic enzymes	+ 7d	+ 13d	+ 4d	+ 4d

b. means a bubble of gas only was formed.

control was not treated with any enzyme and shows an increasing fermentation time. Experience suggests that further transferring would still further increase the time needed to give a positive response.

The portion of the extract treated with DNAase shows a pseudo-transformation in the first Durham tube, the second transfer shows only one bubble of gas and the third tube is entirely negative.

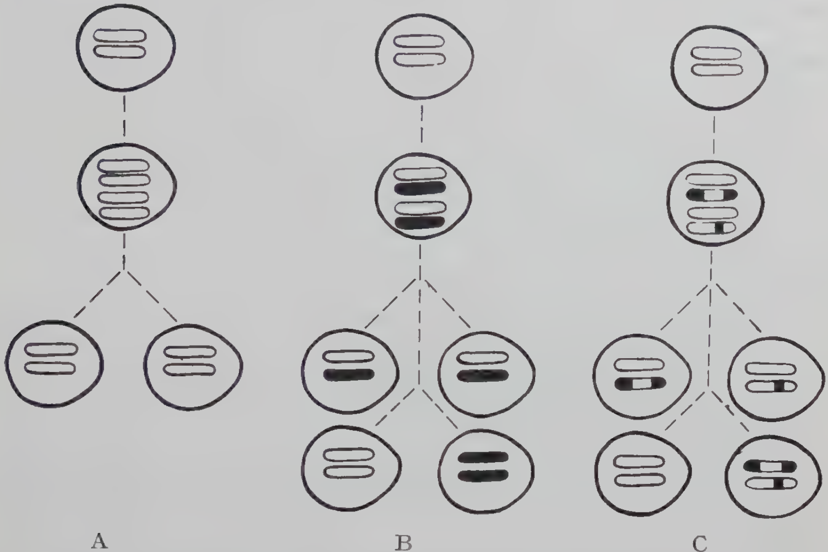
The treatment with RNAase and proteolytic enzymes shows pseudo-transformation as well as a real transformation. From this experiment it can be concluded that DNA is the active agent which induces the real transformation. It may be that the reaction time of half an hour at 30°C. was too short to digest all the RNA and protein matter and some was left in an active state. Further experiments to solve this question are in progress.

VII. Discussion.

In setting up transformation experiments it had to be visualised what could happen. Avery (1944) had shown with bacteria that a transformation could be obtained and later it was proved that DNA was the active substance. It would be of interest to us to know if yeasts also could be transformed and to know where the foreign DNA was localised. The assumption was made that the ingested foreign DNA was localised entirely in the nucleus because it seemed unlikely that DNA would occur elsewhere in the cell. Consequently the DNA molecules would be used when the nucleus was dividing and multiplying. Instead of having to synthesise, the nucleus would accept the ingested molecules as prefabricated material, neglecting, as one might say, the hereditary consequences.

DIAGRAM 4.

DNA working hypothesis.



A. The normal duplication of the chromosomes. For simplicity only one pair of chromosomes per cell is shown. All the new DNA is synthesised.

B. The supply of DNA is abundant, no DNA is synthesised. The new chromosomes are built up of ingested DNA entirely. Each pair of chromosomes divide. Either the two cells of the bottom row or the two cells of the top row originate. Because this happens at random, all 4 types will be found in equal amounts and it therefore looks like a regular mendelian segregation in a F_2.

C. The supply of DNA is limited, only parts of the chromosomes are built up of ingested DNA, the other parts are synthesised by the cell itself. All kinds of combinations of genes wil occur.

In diagram 4 this hypothesis is illustrated. In A the normal division of the nucleus is given. For simplicity only one pair of chromosomes per cell is shown. Before the cell begins to bud the nucleus will divide and multiply. Each chromosome synthesises an identical one. In B an abundant supply of DNA is supposed, here no DNA will be synthesised but the newly formed chromosomes will be built up of the ingested foreign DNA entirely. When the supply is restricted as is supposed in C, only parts of the chromosomes will be built up of the foreign DNA, the other parts will consist of synthesised DNA. During the division from each pair of chromosomes one goes to the left and the other to the right. Either the two cells of the bottom

row or the two of the upper row will originate. This happens at random and therefore in a large number of cells each type will be found in equal amounts. In B an apparent normal mendelian segregation occurs. In the experiments reported here B was not to be expected when the amount of active substance obtained by extraction was rather small. In this respect there was a discrepancy between the preliminary experiments and the later ones. In the former the transformed cells gained the whole complex of characters of the DNA donator including, unfortunately, the poor sporulation and the bad viability of the spores, so that systematic investigation of the genetic make-up was almost impossible.

In later experiments, however, all kinds of combinations of characters occurred as in C (diagram 4). Those results are compiled in table 1. Subculturing several times had no diminishing effect on the fermenting power, on the contrary, the fermentation started earlier. The progeny of the transformed cells obtained by isolation of spores, also possessed the induced characters. The acquired abilities were retained after sexual or asexual reproduction. So it is to be concluded that the acquired abilities were hereditary. The hypothesis given in diagram 4 assumes localisation of the ingested DNA in the nucleus. When it is localised exclusively in the nucleus, the mendelian laws would hold but if divided between the nucleus and the protoplasm, the segregation would be irregular and non-mendelian. To solve this question, in the first place spores of transformed cells were isolated and secondly hybridisation experiments were undertaken. Except for some exceptions, the results with the isolated spores pointed to a normal mendelian segregation.

The ease with which an acquired ability was lost is the only feature which interferes with this interpretation[1]). Most of the isolated transformed cells were heterozygous for the newly acquired abilities. In contrast to the original yeast, some transformed cells could not ferment melibiose. All these facts could be explained by the working hypothesis.

The hybridisation of the transformed cells with each other and the back crosses with the original strain afforded no absolute evidence but made a localisation of the foreign DNA exclusively in the nu-

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[1]) In later experiments it turned out that the acquired ability was not really lost but was inhibited by poor adaptation. Transferring one or two times did give a positive result.

cleus very probable. The working hypothesis, set up before starting the experiments could still be maintained.

Another phenomenon which at first gave rise to some confusion interfered. It has been designated pseudo-transformation and appears as a temporary ability to ferment which diminishes during successive transfers into the same sugar broth. Here the strange phenomenon of de-adaptation in the sugar broth concerned was encountered. When these two phenomena, transformation and pseudo-transformation, could be distinguished clearly, the assumption was made that the former was induced by DNA while the latter was induced by RNA.

This assumption was based on the results of experiments with the ultra-centrifuge. An extract was run and gave a curve with two peaks. The two substances had about the same molecular weight of the order of 10^6 and were about equal in concentration (49,500 r.p.m., temp. 17.5°C., sedimentation time 3.3 and 4.5×10^{-13} in 0.14 M NaCl). Chemical analysis showed the presence of DNA and RNA while protein was practically absent. To test this assumption parts of the extracts were treated with the enzymes deoxyribonuclease and ribonuclease. It was pointed out that the extract contained DNA, RNA and some protein matter and that these substances were not active in the fermentation test. That part of the extract which was treated with DNAase should induce a pseudo-transformation but not a transformation. A treatment with RNAase on the other hand should induce only a transformation.

The results of these experiments did not confirm the hypothesis convincingly and further work is required for a full confirmation. From reports in the literature it seems to be established that DNA causes a heritable change and it is assumed that RNA induces only a temporary change. This would reveal the role of RNA in the cell and give an explanation of the long term adaptation.

The hereditary characters have their foundation in the chromosomes, in the structure of the DNA which is determined by the sequence of the bases. DNA is able to multiply itself and at the same time gives rise to RNA of characteristic pattern. RNA then leaves the nucleus and enters the protoplasm where it is able to synthesise enzymes. But RNA is not able to multiply itself.

When a cell has the chance to take up foreign RNA, it can synthesise the corresponding enzymes but has only acquired the ability temporarily. After each cell division, the amount of foreign RNA is

decreased, less enzyme is formed, fermentation ability diminishes and eventually fades out. This explains the "de-adaptation" in the sugar broths concerned and accords with the RNA template theory of Spiegelman (1957). These experiments demonstrate the possibility of the formation of new enzyme molecules not guided by DNA. In our experiments sometimes a yeast needed an adaption time of say 20 days before fermentation started. Genetically this yeast was able to ferment and in the nucleus a gene for this ability must be present. When no corresponding RNA is present, no enzymes are formed initially and no fermentation is possible. According to Spiegelman (1955) and Monod (1952) the substrate can induce the production of enzymes. That means that the DNA will be stimulated to synthesise the required RNA which in its turn synthesises the enzymes. This arrangement takes time and corresponds to the adaptation time. This explains why a full grown yeast culture after 20 days still showed no fermentation but after 21 days the Durham tube was completely filled with gas. However, the question remains as to why the DNA is stimulated.

When an adaptation time is needed, it means that no enzyme is present and possibly no corresponding RNA. This had a bearing on the possibility of genes for slow fermentation and will be investigated later.

The fermentation of the different sugars needs different enzymes which originate from different genes. The phenomenon of pseudotransformation indicates that different specific RNAs exist and that these can function as specific enzyme templates. It is proposed in future work to attempt a separation of specific RNAs by fractional precipitation.

Summary.

The hybridisation of brewing yeast is almost impossible on account of the poor sporulation, the bad viability of the spores and the failure to conjugate on the rare occasions when viable spores have been obtained.

In experiments by Avery *et al.* with bacteria and by Benoit *et al.* with ducks it was found that administration of nucleic acid preparations induced hereditary modifications. The present report describes the application of an analogous technique in the study of yeast genetics.

Gajdusek's method for the extraction of deoxyribonucleic acid (DNA) gave a very poor yield when applied to yeast and an alternative method giving a good yield is described.

DNA from a brewery yeast conferred ability to ferment certain sugars on a yeast normally deficient in this respect but the cell variants produced, still showed poor sporulation so that a systematic analysis of their genetic constitution could not be made.

In later experiments DNA extracted from *Sacch.chevalieri* was allowed to act on the deficient yeasts and besides acquiring ability to ferment certain sugars the transformed progeny had good sporing characteristics. Four-spored asci were dissected and the characters of the individual spore cultures are described.

Normally the induced transformations were permanent in vegetative reproduction but in some cases a pseudo-transformation was induced in which the newly acquired ability to ferment certain sugars was lost in successive re-inoculations. It was made probable that this pseudo-transformation was induced by ribonucleic acid (RNA) and a theory to explain transformation and pseudo-transformation is advanced.

It would appear that ingested DNA is localised in the nucleus and so mendelian laws will apply. The ingested DNA does not necessarily carry with it the complete character of the DNA donator and the treated yeast will contain cells with all sorts of combinations of the genes in homozygous and in heterozygous state.

It was found that transformed cells could be readily crossed with the original yeast and a preliminary "chemical" hybridisation would thus provide a means of obtaining true stable hybrid varieties of brewing yeasts which might possibly be of technical significance.

The presence of RNA interferes with this process but recognition of its effect reveals something of the mechanism of cell physiology. The results accord with the template theory of Spiegelman and indicate the presence of different ribonucleic acids specific for different enzymes.

Résumé.

L'hybridisation de la levure de brasserie n'est guère possible à cause de la sporulation faible, la mauvaise viabilité des spores et la difficulté de copulation entre les spores ou les premières cellules.

En continuation des travaux d'Avery e.a. avec des bactéries et

ceux de Benoit e.a. avec des canetons on a commencé des expériments avec la levure de brasserie. Les expériments préliminaires avaient du succès. Vu la faible sporulation et la mauvaise viabilité des spores il a été impossible de faire une analyse rigoureuse.

Dans les expériments ultérieurs on a préparé des extraits d'une levure sauvage, *Sacch.chevalieri*. Avec ces extraits on a traité un hybride de levure de brasserie à bonne sporulation et aux spores viables. On a trouvé une nouvelle méthode d'extraction, convenable aux levures. Cette méthode l'a rendu possible d'obtenir une préparation active en partant de 5 ml de levure comprimée (dans les expériments préliminaires on a dû partir d'une quantité de 1 á 10 l de levure comprimée).

Il fut établi que la levure peut être transformée également. La transformation, une modification héréditaire, fut amenée par l'ADN. A côté de la transformation on a découvert un phénomène nouveau qu'on a appelé pseudo-transformation. C'est une modification, héréditaire en apparence, qui pourtant se diminue dans les générations suivantes. Il fut établi que l'agent provoquant cette pseudo-transformation est l'ARN. Ces deux phénomènes se distinguent nettement.

Sur base des résultats obtenus on a établi une théorie permettant d'expliquer la transformation et la pseudo-transformation. Probablement l'ADN digéré se trouve localisé uniquement dans le noyau. Ainsi les lois de Mendel sont encore valables ici. Ce n'est pas nécessairement tout le complexe de caractéres qui est transféré. La levure traitée contient des cellules de toute sorte de combinaisons de gènes, dans l'état homozygote aussi bien que hétérozygote. On a trouvé que le croisement des cellules transformées avec la levure initiale avait plus de succès que celui entre les souches de levure initiale. En tenant compte avec les résultats et la compréhension obtenus dans ces expériences on peut espérer que cette méthode de hybridisation "chimique" permettra d'améliorer la levure de brasserie par sélection génétique moderne.

Pour le présent le rôle de l'ARN n'est pas important pour la pratique, mais il révèle encore un peu du mécanisme de la physiologie de la cellule. Les résultats donnent appui à la théorie de Spiegelman sur l'ARN. En outre, ils suggèrent l'existence de plusiers différents ARN qui sont spécifiques pour différents enzymes.

Zusammenfassung.

Die Hybridisation von Brauereihefe ist nahezu unmöglich durch die schwache Sporenbildung, die geringe Lebensfähigkeit der Sporen und die ungenügende Neigung zur Kopulation der Sporen oder der ersten Tochterzellen.

In Nachfolgung der Versuche von AVERY u.A. mit Bakterien und BENOIT u.A. mit Enten wurden Transformationsversuche mit Brauereihefe angestellt. Die vorbereitende Versuche hatten Erfolg. Mit Rücksicht auf die schwache Sporenbildung und der geringen Lebensfähigkeit der Sporen war es nicht möglich, eine weitere Analyse vorzunehmen.

Bei späteren Versuchen wurden Extrakte aus einer wilden Hefe, *Sacch.chevalieri*, bereitet. Ein aus Brauereihefe gewonnener Bastard mit guter Sporenbildung und Lebensfähigkeit wurde mit den Extrakten behandelt. Es wurde eine neue, für Hefe geeignete, Extraktionsmethode ausgearbeitet. Mittels dieser Methode konnte ein aktives Präparat aus nur 5 ml gepresster Hefe erhalten werden (bei den ersten Versuchen war 1 bis 10 l Hefe benötigt).

Es wurde festgestellt, dass auch die Hefe transformiert werden kann. Die Transformation, eine erbliche Veränderung, wurde von DNA (Desoxyribonukleïnsäure) hervorgerufen. Ausser der Transformation wurde eine neue Erscheinung entdeckt, welche Pseudo-Transformation genannt wurde. Es ist eine scheinbar erbliche Veränderung, welche aber beim nachherigen Fortpflanzen rückgängig gemacht wird. Es wurde festgestellt, dass hier das RNA (Ribonukleïnsäure) das hervorrufende Agens ist. Die zwei Erscheinungen können deutlich unterschieden werden.

Auf Grund der erhaltenen Ergebnisse konnte eine Theorie zur Erklärung der Transformation und der pseudo-Transformation entwickelt werden. Es ist wahrscheinlich, dass das aufgenommene DNA ausschliesslich im Kern lokalisiert ist. Die Gesetze von MENDEL sind hier also auch noch gültig. Es braucht nicht der ganze Komplex von Eigenschaften übergepflanzt zu werden.

Die behandelte Hefe wird alle mögliche Genenkombinationen, im homozygotischen und heterozygotischen Zustand, enthalten. Es wurde gefunden, dass die Kreuzung der transformierten Zellen mit der Originalhefe besseren Erfolg hatte, als bisher mit der Kreuzung zwischen Stämmen der Ausgangshefe erzielt wurde. Auf Grund der Ergebnisse und der Einsicht, welche mit diesen Versuchen erhalten

wurden, wird die Annahme geäussert, dass diese Methode der "chemischen" Hybridisation einen neuen Weg zur Verbesserung der Brauereihefe mittels moderner genetischer Selektion eröffnen wird.

Augenblicklich ist die Rolle des RNA wahrscheinlich von wenig Bedeutung für die Praxis, sie enthüllt aber etwas mehr über den Mechanismus der Zellphysiologie. Sie geben aber der Theorie Spiegelman's über das RNA weitere Stütze. Des weiteren sprechen sie für das Vorkommen verschiedener RNA, welche für verschiedene Enzyme spezifisch sind.

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DERIVATION OF NON-ACETIFYING „QUASI-ACETOBACTERS" FROM A TRUE *ACETOBACTER* STRAIN, AND *VICE VERSA*

by

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Introductory.

In previous papers (Shimwell 1957, 1959; Shimwell and Carr 1958) two speculative prognostications were made. The first was that, owing to the extraordinary facility with which most *Acetobacter* strains were found to give rise to spontaneous mutants which had lost or gained some major biochemical property, it could not be precluded that even the ability to oxidize alcohol to acetic acid (the main generic character of *Acetobacter*) might be capable of similar loss, although no such mutation had then been encountered.

The other tentative suggestion was that the classical pleomorphism found in the genus *Acetobacter*, and stressed for so long in the literature, might indicate a complex type of reproductive mechanism other than simple fission. It was shown that the large swollen cells found in, *e.g.*, a strain of *A. mesoxydans*, often contained numerous small rod-like granules which, it appeared, might be liberated by the rupture of the large bodies to fulfil some reproductive function (Shimwell 1959).

In this paper we offer experimental evidence which seems to confirm the s i m u l t a n e o u s occurrence of both the above mentioned phenomena, and thus rather suggests that they may be connected.

Definitions.

Great difficulty has been found in deciding what terms to apply to the various swollen cells or bodies, and also to the "mutants" or

"variants" which have lost the power of producing acetic acid and yet do not fit into any other known genus. For the sake of brevity we therefore submit the following definitions of the terms used throughout the ensuing paper.

"Mutation" and "Mutant". These terms are used in a broad sense to refer to all heritable changes of characteristics, whether these have been due either to changes in "nuclear genes" or in "plasmagenes" or to some other cause.

"Species". Although it has been shown that almost all *Acetobacter* species give rise to mutants corresponding to other *Acetobacter* species (Shimwell 1959) this term is again used for the sake of brevity, and Frateur's (1950) criteria, diagnoses, and classification are adopted.

"Quasi-Acetobacter". This term is used (for lack of a better one) to denote the mutants which have lost the power of acetifying alcohol, and yet cannot be allotted to any existing genus.

Gender of "Acetobacter". In accordance with the 7th edition of Bergey's Manual (1957) we are now treating this as masculine, instead of the previous neuter.

Experimental.

To test a strain corresponding biochemically to *A. rancens* (Frateur 1950) for its ability to produce cider-vinegar, a submerged aeration apparatus similar to that described by Shimwell (1954) was assembled, and all parts autoclaved. It was then filled with Seitz-filtered cider, and filtered and metered air was bubbled through it by means of a sintered glass gas-distributor. It was then inoculated with a culture of the *A. rancens* strain (which was of reputable extramural origin).

After proliferation of the bacteria had commenced, four successive acetifications up to an acetic acid content of 4% (w/v) were carried out. During the last acetification the liquid was plated out in serial dilution by spreading on apple-juice agar.

In view of previous results (Shimwell 1956), using the same process, it was not altogether surprising to find that several different colony forms were obtained. What was indeed surprising was that most of the colonies obtained yielded cultures no longer capable of producing acetic acid from alcohol, thus apparently having mutated out of the genus *Acetobacter*, altogether.

Contamination of the culture used was, of course, at first suspec-

ted, as control samples of the Seitz-filtered cider, incubated without inoculation, had remained sterile. The parent strain of *A. rancens* used for seeding the acetifier was therefore examined by plating on unhopped-beer agar containing 4% (w/v) alcohol, and adjusted to pH 6 to ensure firm setting, this medium having been found to show up mutant colonies very well.

The number of different colony forms developing equalled and even surpassed that found in previous mutation studies (SHIMWELL 1957, 1958 and 1959). A striking feature of the plates was, however, the presence of many minute colonies invisible to the naked eye and only just detectable with a magnification of × 40, using oblique transmitted light (fig. 1) [1]).

After 48 hours at 27° C. they seemed to cease to grow any larger, but after a further 4 days' incubation they began to increase in size, eventually yielding medium to large colonies some of which resembled those which had already grown to that size; others were of new colony types.

In the meantime those large colonies which had developed earlier soon began to develop papillae, secondary colonies, outgrowths, or sectors. These arose from various parts of the parent colony, sometimes being horizontally peripheral, sometimes developing on top of the parent colony (fig. 2). After about 7 days' incubation the picture presented was that of a mixture of large, small, and medium-sized colonies of different types, many with secondary outgrowths or papillae resembling other colonies, or being new colony types.

The original dwarf colonies, soon after they had commenced to increase in size, themselves began to develop outgrowths, papillae, etc. which, in turn, developed into colonies different from the dwarf parent, and so on, and so on (fig. 3).

Attention is also drawn to the development of small rhizoid outgrowths from many of the larger original colonies. These outgrowths were very similar in appearance to the original separate dwarf rhizoid colonies. In fig. 4 one of these rhizoid outgrowths can be seen arising from the periphery of a large mutating colony; note also the serrated margin of the separate dark circular colony, as distinct from the smooth margin of the other.

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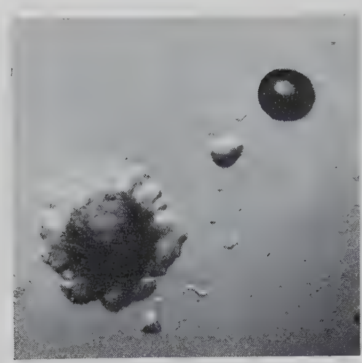
[1]) In a previous study of an *Acetobacter* strain producing swollen granule-containing bodies (SHIMWELL 1959) which possibly liberated rodlets, no half-expected dwarf colonies suggestive of L-forms or similar phenomena were observed; this time these were much in evidence.



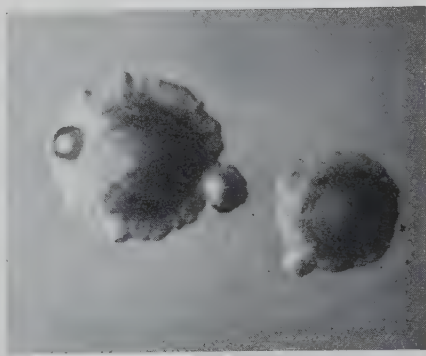
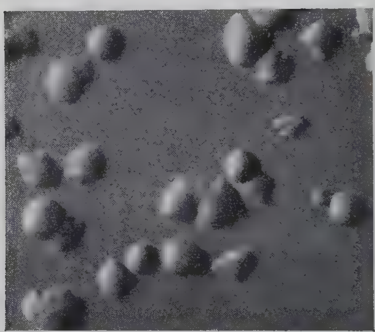


Fig. 1. Dwarf and "normal" colonies from parent *A. rancens* strain. × 40.
Fig. 2. Papillae and outgrowths on "normal" colonies. Parent *A. rancens*. × 40.



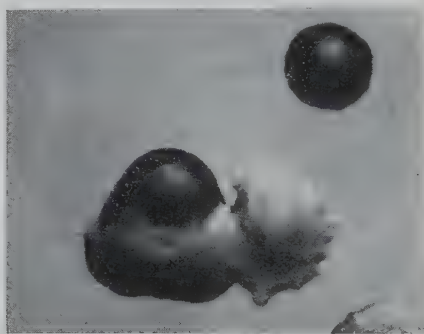


Fig. 3. Developing and mutating dwarf colonies from parent *A. rancens* strain. × 40.

Fig. 4. Small rhizoid outgrowth from mutating large colony of parent *A. rancens* strain. × 40.

Biochemical Properties of the Different Colonies.

The original object of this investigation, was, however, to see if the culture used for inoculating the vinegar generator contained "quasi-acetobacter" strains corresponding to those which had apparently outgrown the true *Acetobacter* cells in the course of the acetification. Eight young, well-isolated colonies, devoid of papillae, sectors, or outgrowths, and all of markedly different colony form, were picked and transferred to separate slopes of YE-ethanol-agar, containing brom-cresol-green as internal indicator. Of these, 4 proved to be acetic acid bacteria, and 4 quasi-acetobacters, cultures of the latter remaining unchanged in tint after prolonged (4 weeks') incubation at 27° C., the *Acetobacter* cultures turning yellow in 18 to

24 hours. The properties of all the strains obtained from both plates and acetifier are shown in Table 1. FRATEUR's methods (1950) were used.

TABLE 1.

Biochemical properties of colony mutants of *A. rancens* parent.

	P	1	2	3	4	5	6	7	8	9
Alcohol to acetic acid	+	0	+	0	0	+	+	+	0	0
Acetate to carbonate	+	+	+	+	+	+	+	+	+	+
Lactate to carbonate	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Growth on Hoyer's medium	0	0	0	0	0	0	0	0	0	0
Acid from glucose	+	+	+	+	+	+	+	+	+	0
Dihydroxyacetone	0	0	0	0	0	0	0	0	0	0
Cellulose	0	0	0	0	0	0	0	0	0	+
Starch	0	0	0	0	0	0	0	0	0	0
Colony form	N	ST	SO	R	RH	SO	Z	R	R	SO
Colony texture	B	C	B	C	B	B	B	B	C	V
Motility	0	0	0	0	0	0	0	0	0	+

P = parent strain. + = positive; 0 = negative. N = numerous. ST = smooth translucent. SO = smooth opaque. R = rough (various types). RH = rhizoid. Z = zoned. B = buttery. C = creamy. V = viscid.
(No. 9 was isolated directly from the acetifier after four acetifications).

The first notable point is that all quasi-acetobacter strains, whilst failing to acetify alcohol (confirmed on YE-ethanol-$CaCO_3$ oxydogram), nevertheless possess the power of oxidizing calcium lactate and acetate to carbonate. It will also be noted that isolate 9 (a quasi-acetobacter) differs in two properties from all the other isolates. It has lost the power of producing acid from glucose, and gained the power of producing cellulose (relatively common mutations in *Acetobacter* proper).

This isolate was also motile, with peritrichous flagella. (An occasional motile cell was visible in the parent *A. rancens* strain). The "cellulose" was not leathery like that of *A. xylinum*, but was of the "soft" type (SHIMWELL and CARR 1959); nevertheless the cellulose reaction was very definite.

POSSIBILITY OF CONTAMINATION.

The above evidence, although strongly supporting the hypothesis that the quasi-acetobacter strains encountered were derived from

the original *Acetobacter rancens* strain, does not constitute absolute proof of this. Nevertheless the probability of an *Acetobacter* culture in acid media becoming contaminated with other previously unknown, strictly aerobic, morphologically and culturally similar bacteria, differing from acetobacters in only one detectable characteristic — namely the inability to acetify alcohol — is remote for a number of reasons, two of which are as follows:-

1. A medium of pH 3.5, particularly if that pH has been attained by means of acetic acid (the most bactericidal of all common acids) is an extremely selective one, eliminating most bacterial types other than *Acetobacter* strains. These quasi-acetobacters, moreover, could not only grow in such conditions but could oxidize the acetic acid to CO_2 and H_2O, so that the pH rose. Apart from *Acetobacter* we know of no genus containing bacteria capable of the above activity under such conditions.
2. Although the colonies produced both by the quasi-acetobacters and the acetobacters were many and various, it was found that some colony forms were common to both types. In one instance two colonies of identical appearance were picked; one proved to be an acetobacter, the other a quasi-acetobacter.

However, stronger evidence, virtually amounting to proof, provided itself in the following way.

BACK-MUTATION.

One quasi-acetobacter isolate (No. 1), after serial transfer, began to show feeble signs of producing acid on YE-alcohol-agar-slopes. This tendency increased on further sub-cultivation. Upon then plating this culture it was a comparatively easy matter to isolate both acetobacters and quasi-acetobacters, the feeble acetifying power gained not being due to a gain of that property by a l l the cells, but probably to the production, by back-mutation, of a mixture of cells, some strongly positive, the majority completely negative.

Conversely, an example of mutation of one of the *Acetobacter* isolates to yield a quasi-acetobacter was also obtained. *Acetobacter* No. 6 gave rise, after serial transfer, to a few quasi-acetobacter colonies. Some of these, after further separate cultivation and replating, subsequently back-mutated to yield acetobacters again.

In contradistinction to this, quasi-acetobacters Nos. 4 & 9 have

not, so far, gained the ability to produce acid from alcohol, being apparently stable quasi-acetobacter strains.

Repetition of Acetification Experiment.

The submerged-aeration acetification of cider described in the first part of this paper was repeated, using even more thorough autoclaving, and taking even more rigorous precautions to ensure sterility, and rule out the possibility of contamination. In the course of the very first acetification the air supply was inadvertantly interrupted for a period of about 30 minutes. As found by Hromatka and Ebner (1948) and subsequently confirmed by us on numerous occasions, such interruptions of air supply under submerged aeration conditions usually results in the virtual "destruction of the culture", acetification failing to resume (or resuming only after several days) when the aeration is continued. On this occasion, however, as a matter of interest, aeration was continued after the interruption. It was found, as expected, that no further increase in acid took place. But, on the contrary (and unexpectedly) the acetic acid content rapidly began to d i m i n i s h , at a rate rising to about 1% (w/v) per 24 hours. On plating the "over-oxidizing" liquid almost all the colonies were found to be of quasi-acetobacter strains.

It is suggested that the ability of quasi-acetobacters apparently to survive conditions of little or no aeration accounted for their predominance in the cider acetification described. Furthermore, it is extremely difficult to supply exponentially multiplying, obligatively aerobic, acetic acid bacteria, in the submerged process, with sufficient air to meet their rapidly increasing requirements. For this reason many such acetifications probably suffer, throughout the whole period, from a shortage of all the air really required. This would account for the presence of quasi-acetobacters in the previous acetification experiment in which there was no actual i n t e r-r u p t i o n of the aeration.

A Starch-Producing Quasi-Acetobacter.

The production of voluminous extra-cellular mucilage giving an almost instantaneous dark blue coloration with simple iodine solution appears to be rare amongst bacteria other than *Acetobacter pasteurianus*. (*A. kuetzingianus* is regarded, with Frateur (1950), as a mere cultural variety of *A. pasteurianus*).

One of us (J.G.C.) has frequently encountered starch-producing

Acetobacter strains in cider. From one particular sample of cider all the isolates obtained proved to be strong starch-producers. Unexpectedly, however, one of these starchy isolates did not produce acetic acid from alcohol, although it oxidized acetate to carbonate. Minute rhizoid colonies were again a feature on plates.

On sub-cultivation and subsequent replating on apple-juice agar, however, two true *Acetobacter* mutants were obtained with properties shown in Table 2.

TABLE 2.

Properties of acetobacters derived from a starchy quasi-acetobacter.

	Parent	Mutant 1	Mutant 2
Alcohol to acetic acid	0	+	+
Acetate to carbonate	+	+	+
Lactate to carbonate	+	+	+
Catalase	+	+	+
Growth on Hoyer's medium	0	0	0
Dihydroxyacetone	0	0	0
Acid from glucose	0	+	+
Cellulose	0	0	0
Starch	+	0	+
"Species"	?	*A. rancens*	*A. pasteurianus*

The fact that this quasi-acetobacter gave rise to mutants indistinguishable from two classical *Acetobacter* species, considered in conjunction with the similar phenomena previously described in this paper, seems to lead to the conclusion that there is a further mutable criterion to be taken into account when assessing how many "species" are possible (but species of what?). Instead of the 5 criteria of Frateur (which makes the number of possible species 32) there now seem to be 6, giving 64 possible "species". Indeed, in the course of this single investigation there are already several towards the total number — all quasi-acetobacters unclassifiable in any genus if they had been isolated from a natural habitat by someone unfamiliar with the acetic acid bacteria.

Cell Morphology.

a. Parent *A. rancens* strain. This culture was notable for the presence of large numbers of the long swollen filaments and large bodies traditionally mentioned in the literature. Many of these

large bodies appeared to have a granular internal structure when examined in wet mount, a phenomenon previously described and illustrated in the case of an *A. mesoxydans* strain (SHIMWELL 1959).

Such swollen bodies have been described by most workers as occurring only or mainly in old cultures, but, as FRATEUR (1950) has noted (and we have confirmed) such "involution" forms may be found in cultures as young as 24 to 36 hours (fig. 5).

Fig. 5. Large swollen cells in parent *A. rancens* strain. Negative stain. × 1,000.

b. Quasi-acetobacter No. 1. This culture contained virtually as many swollen filaments and large bodies as the parent *A. rancens* culture, a large proportion of the total cell population consisting of this type. Upon plating this culture, a dilution estimated by visual count to give about 300 colonies per plate did give this number of "normal" colonies; but, in addition, there were some thousands of minute rhizoid or elongated colonies between the larger ones. Fig. 6 illustrates this phenomenon. One cannot help wondering whether these innumerable dwarf colonies might have originated from the granular contents hypothetically liberated from ruptured large bodies.

c. Acetobacter No. 6. This culture also contained a large number of very swollen long filaments. On plating this, many dwarf rhizoid colonies were again found, but these, unlike those in quasi-acetobacter No. 1, slowly increased in size. On picking some of these

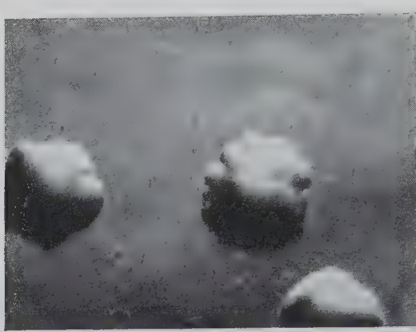


Fig. 6. Numerous dwarf rhizoid colonies from quasi-acetobacter No. 1. × 40.

developing dwarf colonies, some were found to be acetobacters and some to be quasi-acetobacters. Furthermore, some of the latter, on replating, often back-mutated to yield acetobacters.

d. Acetobacters Nos 2, 5 & 7. These did not contain any swollen filaments, although the "normal" cell-form varied from short to very long, even in the same culture. However, this is what is often found in many classical *Acetobacter* strains.

e. Quasi-acetobacters Nos 4 & 9. These did not contain any filaments or large bodies. It is interesting to note that these two isolates have not, so far, shown any signs of back-mutating, or change of characteristics.

f. Quasi-acetobacters Nos 3 & 8. These contained short and medium rods and some filaments, although the latter were not so swollen as in other cases.

Apparent Correlation of Cell Form with "Instability".

It so happens that those cultures (whether acetobacters or quasi-acetobacters) which contained swollen filaments have been those which yielded unstable cultures which "mutated" one way or the other. The cultures of relatively normal cells (*i.e.* 2, 4, 5, 7, 8 and 9) gave rise to cultures which, whether quasi or true acetobacters, remained so, being apparently stable (or at least as stable as are most *Acetobacter* strains). This apparent correlation between the presence of swollen bodies and mutation (if it *is* mutation) may be merely coincidental, as the number of cultures examined is, of course, small; nevertheless the coincidence seems noteworthy.

Discussion.

The first significant point in connection with the foregoing results and observations is the apparent correlation between the presence, in the parent strains, of large swollen cells, the development of numerous dwarf colonies when such cultures were plated, and the isolation, from such plates, of quasi-acetobacters from the true *Acetobacter* parents or *vice versa*. The abundance of such swollen forms, and the associated phenomena, tempts one to put forward some speculative explanation of their function. At the moment, however, the data, although abundant, are so confusing and apparently disconnected that we feel there is no justification for doing more than merely recording our observations and results.

On the other hand there seems nothing vague or indefinite about the existence of "quasi-acetobacters", which are not only derivable from true acetobacters, but possess all the properties of one species or another of *Acetobacter*, except the very one necessary for their continued inclusion in that genus — namely the all-important production of acetic acid from alcohol. Conversely, an independently isolated quasi-acetobacter, possessing the rare power of producing voluminous extra-cellular starch, can give rise to a true *Acetobacter* "species" also producing such starch, and corresponding to one of the earliest *Acetobacter* species to be described by Hansen (1879) namely *Mycoderma pasteurianum* (*A. pasteurianus*).

We have obtained considerable evidence (although not absolute proof, so far) that when once an *Acetobacter* culture contains quasi-acetobacters derived from it, a state of symbiosis may be established, the acetobacter producing acetic acid which the quasi-acetobacters can then oxidize to CO_2 and H_2O, whilst, in return, the quasi-acetobacters, by removing some of the acetic acid as it is formed, benefit the acetobacter by preventing the acid content and/or pH from reaching an inhibiting value. True *Acetobacter* strains usually cannot do this for themselves, being unable to oxidize the acetic acid they have produced until all the alcohol is gone; then they can often do so readily.

It seems to us that the existence of these quasi-acetobacters and their derivation from true acetobacters, together with their ability themselves to give rise to the latter, makes the foundation of the genus *Acetobacter* somewhat shaky, if it does not virtually demolish it altogether. In the first place, to abolish the generic criterion of acetic acid production from alcohol, thus putting acetobacters and

quasi-acetobacters on an equal taxonomic footing, would involve transferring this then vague combined group to some other equally vague and ill-defined genus, such as *Achromobacter*.

Alternatively it may be that quasi-acetobacters, in their natural habitat, may be related to, *e.g.*, other non-acetifying bacteria associated with, say, fruit or fermenting vegetable matter, a suggestion which is, of course, purely speculative.

Proposed Further Work.

It is considered that the apparent correlation of swollen filaments and the occurrence of quasi-acetobacters may possibly be of importance both from the evolutionary and the taxonomic point of view. However, the continuation of this work on lines similar to those recorded in this paper, whilst no doubt capable of yielding further unusual observations, does not seem likely to lead to elucidation.

In this connection Prof. Ø. Winge and, independently, Dr. F. W. Beech (private communications) have suggested that the isolation of single cells, particularly the large bodies, using a micromanipulator, might be the best, and perhaps only, method of attack of the problem, and it is hoped to follow up this suggestion. On the other hand, more requires to be known of the biochemical activities of the quasi-acetobacters in the hope of finding some clue to their relationship (if any) to some other more naturally occurring group of peritrichously flagellated bacteria.

Availability of Cultures.

The following freeze-dried cultures are available at the Research Station, Long Ashton:

a. The original parent strain of *A. rancens*.
b. Quasi-acetobacter No. 4 (non-cellulosic).
c. Quasi-acetobacter No. 9 (cellulosic).

A starch-producing quasi-acetobacter and a non-starchy one, both derived from a common quasi-acetobacter parent, have been deposited with the National Collection of Industrial Bacteria, Teddington, Middlesex, where they are numbered respectively 8956 (starchy), and 8957 (starchless).

Those who care to study these strains are advised to do so immediately after reviving the freeze-dried cultures, as these strains may

be highly "mutable" and some may soon change their characteristics during serial transfer.

Summary.

A strain of *Acetobacter rancens* gave rise to "quasi-acetobacters" which had lost the essential generic character of oxidation of ethanol to acetic acid, whilst retaining all the other characteristics of the genus. Other quasi-acetobacters with other acetobacter properties were also obtained. Conversely a starch-producing quasi-acetobacter gave rise to two true *Acetobacter* strains indistinguishable biochemically from *A. pasteurianus* and *A. rancens* respectively.

These phenomena were associated with the presence, in the parent strains, of many swollen filaments and large bodies. It is tentatively suggested that the changes from true to quasi-acetobacter, and *vice versa*, may be correlated with the production of such bodies, and may indicate some heterodox form of reproduction other than simple fission.

The advent of "quasi-acetobacters" seems largely to demolish the genus *Acetobacter*, whilst failing to indicate an alternative under the existing rigid botanical taxonomic conventions.

Acknowledgements.

We wish to acknowledge our indebtedness to Prof. Ø. WINGE for his valuable opinion and suggestions concerning the possible nature of the large granule-containing bodies; to Dr. C. B. VAN NIEL for stimulating correspondence with one of us (J.L.S.) concerning the difficulty of classifying *Acetobacter* strains; and to Dr. H. LÜTHI for the parent *A. rancens* strain used in this investigation.

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A NEW SPECIES OF *SCHWANNIOMYCES*: *SCHWANNIOMYCES ALLUVIUS*

by

H. J. PHAFF, M. W. MILLER and Wm. BRIDGE COOKE

(Received October 30, 1959).

The genus *Schwanniomyces* was established by KLÖCKER in 1909 to include the single species, *S. occidentalis* Klöcker, isolated from a soil sample obtained on the island of St. Thomas in the Virgin Islands, West Indies. LODDER and KREGER-VAN RIJ (1952) essentially confirmed KLÖCKER's diagnosis and maintained the genus which contained a single species. CAPRIOTTI (1957) described a second species, *S. castellii,* of which two strains were isolated from the soil of a vineyard near Madrid, Spain. This species differed from *S. occidentalis* in its ability to assimilate lactose and to ferment galactose (rather weakly) and maltose (very weakly). In addition, *S. castellii* formed predominantly spherical cells, whereas *S. occidentalis* developed more ovoid cells.

During a survey of fungi associated with waste disposal systems, two yeast strains were isolated which appear to constitute a new species in the genus *Schwanniomyces*. These cultures were isolated from moist soil of the bank of Lytle Creek, Clinton Co., Ohio, from two locations about one mile apart. We propose to name the new species *S. alluvius* since this specific name indicates the type of soil from which it was isolated.

Standard description of *Schwanniomyces alluvius* nov. spec.

Growth in malt extract: After 3 days at room temperature, cells sub-spherical to broad ovate, $(4.0\text{-}8.8) \times (5.3\text{-}11.0)\mu$; mainly in short chains or clusters; slight ring and sediment formed. After 3 weeks ring and sediment present.

Streak culture on malt agar after one month: light cream colored with smooth, semi-glossy surface; texture pasty; cross section low convex (spreading); border entire.

Slide culture on potato-glucose agar: pseudo mycelium absent.

Sporulation: numerous asci formed on yeast autolysate agar with 2% glucose after 5-7 days. Spores warty, spherical to globose with an equatorial ledge and a pronounced lipid globule in the center; usually one, occasionally two spores per ascus; one or two bud-like structures attached to ascus by a wide neck (fig. 1). After isogamic conjugation asci arise very rarely.

Fermentation:		
	glucose +	maltose + (latent)
	galactose + (moderate)	lactose —
	sucrose +	inulin +
	raffinose + 1/3	soluble starch + (weak)

Assimilation: Positive: glucose, galactose, sucrose, maltose, cellobiose, trehalose (latent, weak), melibiose, raffinose, melezitose (latent), inulin, soluble starch, D-xylose, L-arabinose, ethanol, adonitol (latent, weak), D-mannitol, D-sorbitol, alpha-methyl-D-glucoside, salicin (weak) and Ca-2-keto-gluconate.

Negative: L-sorbose, lactose, D-arabinose, D-ribose, L-rhamnose, glycerol, i-erythritol, dulcitol, K-5-keto-gluconate, DL-lactic acid, succinic acid, citric acid, i-inositol, and glucono-δ-lactone.

Assimilation of $(NH_4)_2SO_4$: positive; of KNO_3 and KNO_2: negative.

Growth in a vitamin-free medium: negative.

Type strain: 54-83 (SEC 1310). Additional strain: 54-103 (SEC 1466).

Habitat data: SEC 1310 isolated from bank soil, always subject to flooding, at EHC station 2.8, upper part of lower clean water zone, Lytle Creek, Clinton Co., Ohio. July 10, 1952.

SEC 1466 isolated from bank soil, always subject to flooding, at EHC station 5.2, lower recovery zone, Lytle Creek, Clinton Co., Ohio. Sept. 18, 1952.

Latin diagnosis:

Schwanniomyces alluvius sp. nov.

In musto maltato cellulae rotundae aut ovoidae, (4.0-8.8 × 5.3-11.0 μ) singulae binae aut catenatae; sedimentum et anulus formantur. In agaro maltato cultura albida glabra, semi-nitida, mollis, margine glabro. Pseudo-

Fig. 1. Asci of *S. alluvius* showing typical ascospores and meiosis buds.

mycelium nullum. Ascosporae rotundae aut ovoidae, verrucosae, anulum in medio, 1-2 in asco. Fermentatio glucosi, galactosi (leniter), sacchari, maltosi (lente), raffinosi pro tertia parte, inulini, amyli (exigui). In medio minerali cum glucoso, galactoso, saccharo, maltoso, cellobioso, trehaloso (exiguo), melibioso, raffinoso, melezitoso (exiguo), inulino, amylo, D-xyloso, L-arabinoso, alcohole aethylico, adonitole (exiguo), D-mannitole, D-sorbitole, alpha-methyl glucoside, crescit. Nitras kalicos non assimilatur. Necessariae ad crescentiam sunt vitaminae externae.

Since an extended study of the carbon assimilation abilities of the other two species of the genus has not been reported in the literature, a comparative study was undertaken. The results are given in Table 1. Since the ability to ferment various sugars has not always been studied under identical conditions, we also compared the fermentation of various sugars by the three species in Durham tubes. The results for 9 sugars are given in Table 1.

Discussion.

The results reported from tests with *S. occidentalis* agree with those obtained by Lodder and Kreger-van Rij (1952) except that we noted a latent and weak fermentation of galactose and a positive assimilation of ethanol. In tests with galactose the inverted vial became approximately half filled with gas in 8-14 days depending on the amount of inoculum. *S. occidentalis* differs from the other two species in several assimilation reactions and in its inability to ferment maltose (see Table 1).

Observations on *S. castellii* generally confirmed those made by Capriotti (1957) except that maltose was fermented moderately well, a Durham vial full of gas was obtained in 4-5 days, rather than "very weak". Inulin was fermented well, producing a full vial of gas in about 3 days, rather than showing a negative reaction. The assimilation of ethanol was found to be positive rather than very weak.

The new species being described in this paper, *S. alluvius*, is similar to *S. castellii* from which it is differentiated principally by its inability to assimilate lactose.

On the basis of the above study the following key is presented to the species of the genus *Schwanniomyces*:

TABLE 1. Assimilation or fermentation of various carbon sources by species of *Schwanniomyces*.

Species	*S. occidentalis* (type strain)		*S. castellii* (2 strains)		*S. alluvius* (2 strains)	
Assimilation or Fermentation (when tested)	Assimilation	Fermentation	Assimilation	Fermentation	Assimilation	Fermentation
Carbon Source						
Glucose	+	+	+	+	+	+
Galactose	+	LW	+	+L	+	+L
L-sorbose	—		—		—	
Maltose	+	—	+	+L	+	+L
Sucrose	+	+	+	+	+	+
Cellobiose	—		+		+	
Trehalose	LW		LW		LW	
Lactose	—	—	+	—	—	—
Melibiose	—	—	+	L or VW	+	— or VW
Raffinose	+	+W	+	—	+	+
Melezitose	+L		+		+L	
Inulin	+	+W	+	+	+	+
Soluble Starch	+	—	+	LW	+	LW
D-xylose	+		+		+	
L-arabinose	+		+		+	
D-arabinose	—		—		—	
D-ribose	—		—		—	
L-rhamnose	—		—		—	
Ethyl alcohol	+		+		+	
Glycerol	—		—		—	
i-erythritol	—		—		—	
Adonitol	—		LW		LW	
Dulcitol	—		—		—	
D-mannitol	L		+		+	
D-sorbitol	—		+		+	
Alpha-methyl-D-glucoside	—		+		+	
Salicin	—		W		W	
Glucono-δ-lactone	—		—		—	
Ca-2-keto-gluconate	+		+		+	
K-5-keto-gluconate	—		—		—	
DL-lactic acid	—		—		—	
Succinic acid	—		—		—	
Citric acid	—		—		—	
i-inositol	—		—		—	

Key: + growth or fermentation.
— no growth or fermentation.
L latent growth or fermentation.
W weak growth or fermentation.
+L latent positive growth or fermentation.
LW latent weak growth or fermentation.
VW very weak growth or fermentation.
+W positive but weak growth or fermentation.

1. a. Lactose assimilated . . . . *S. castellii*
   b. Lactose not assimilated . . . . (2)
2. a. Maltose not fermented, melibiose not assimilated . . . . . *S. occidentalis*
   b. Maltose fermented (latently), melibiose assimilated . . . . *S. alluvius*

The life cycle of *S. alluvius* has been worked out by FERREIRA and PHAFF (1959). They showed that the cells are haploid and that karyogamy occurs in the ascus mother cell. Meiosis occurs in the bud-like appendages of the asci and the name "meiosis buds" for these structures was suggested. In the older literature these structures had been termed abortive conjugation tubes. The ascospores germinate directly into haploid vegetative cells.

It is of interest that all cultures representing the various species of *Schwanniomyces* were isolated from soil. Since thus far relatively few strains have been isolated, it cannot be stated with certainty that soil is the natural habitat of this genus. As the morphology of the colonies of the different species of the genus is not very characteristic, it is possible that, in the presence of yeasts with similar colonies, those of *Schwanniomyces* may have gone undetected on various isolation media. Since all three species have been shown to assimilate inulin, and this polysaccharide is utilized by few species of yeast, it may be useful to employ a synthetic medium with inulin as the sole carbon source for the plating of various natural substrates presumably containing yeast. The presence of conspicuous lipid globules in vegetative cells of *Schwanniomyces* could be used as an aid in recognizing colonies representing cultures of this genus.

Summary.

A description has been given of a new species of yeast, *Schwanniomyces alluvius*. It was isolated from moist soil of a creek bank in Ohio. The physiological properties of the new species have been compared in detail with those of the two known species of the genus.

Acknowledgment.

The authors express their appreciation to Mr. W. L. MARCUS for taking the photomicrographs of the ascospores.

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HEMOLYTIC EFFECT OF A GLYCOLIPID PRODUCED BY *PSEUDOMONAS AERUGINOSA*

by

G. SIERRA

(Received November 13, 1959).

1. Introduction.

A crystalline acid glycolipid active on *Mycobacterium tuberculosis* has been isolated from *Ps. aeruginosa* by Jarvis and Johnson (1949). These authors investigated its chemical structure, and found it to be composed of two mol. l-rhamnose and two mol. 1-β-hydroxy-decanoic acid linked as follows:

$$\text{l-rhamnose} \xrightarrow[\text{linkage}]{\text{1.3 glycoside}} \text{l-rhamnose} \xrightarrow[\text{linkage}]{\text{1. glycoside}} \text{1 } \beta\text{-hydroxy-decanoic acid} \xrightarrow[\text{linkage}]{\text{ester}} \text{1 } \beta\text{-hydroxy-decanoic acid.}$$

This compound was also synthesized by *Ps. aeruginosa* from glycerol in a mineral medium (Hauser and Karnovsky, 1954). These investigators emphasize the importance of this compound since it represents not only a unique example of a crystalline glycolipid but also might be of interest for a study of the synthesis of its components. The β-hydroxy-decanoic acid is important as an intermediate in the mechanism of lipid metabolism (Lynen, 1953).

The toxicity of the glycolipid to mice was investigated by Jarvis and Johnson (1949) who found that 5 mg given intraperitoneally killed the mice in about sixteen hours.

The present communication deals with the hemolytic effect of the glycolipid.

2. Experimental.

For the production of the glycolipid a strain of *Ps. aeruginosa*

was used. The behaviour of the strain in routine tests is reported elsewhere (SIERRA and ZAGT, 1960). It was cultivated in the same way as reported by JARVIS and JOHNSON (1949).

a) Isolation of glycolipid.

The isolation and purification of the crystalline compound (m.p. 86° C.) was also accomplished in the way described by JARVIS and JOHNSON (1949). Its composition and properties were in agreement with those found by these authors. Data of the elementary analyses are given below:

Analyses[1]: Calculated for $C_{32}H_{60}O_{14}$: C, 57.46; H, 9.04; O, 33.49
Found: C, 57.71; H, 9.02; O, 33.16

The solution of the sodium salts was made by neutralizing a distilled water suspension of the acid glycolipid with 0.1 N aqueous NaOH solution. The pH of the solution was 7.6.

b) Hemolysis test.

Hemolysis was studied in fresh suspensions of goat erythrocytes. To 0.15 ml of washed red blood cells in micro-assay tubes was added 1 ml of the sodium glycolipid solution in normal saline. After centrifuging at 5000 r.p.m. for 15 min. hemolysis was observed.

c) Isolation of cell walls of erythrocytes.

Cell walls of erythrocytes were isolated by hemolysing red blood cells by repeated centrifugation with distilled water and then a suspension in water was made. The isolated materials remain stable in suspension for considerable periods of storage.

3. Results and discussion.

In agreement with the work of JARVIS and JOHNSON (1949) 5 mg of the Na-salt of glycolipid given intraperitoneally killed mice in about six hours.

It might be expected, that the glycolipid may cause a withdrawal of calcium from the organism because of the insolubility of its calcium salts in water.

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[1]) The carbon, hydrogen and oxygen micro-analyses were carried out by Mr. P. J. HUBERS of the Micro-analytical Department, Laboratory of Organic Chemistry, Municipal University of Amsterdam.

However, to judge from the quick and quiet death of the mice, it seems improbable that the removal of calcium is responsible for the high toxicity of the glycolipid. In order to furnish an explanation of this fact, the following experiment was carried out.

To 0.15 ml washed erythrocytes was added 1 ml of a 2% solution of sodium glycolipid in normal saline (0.9% NaCl). After centrifuging it appeared that the dark red content of the erythrocytes had leaked out in the solution.

When this experiment was compared with an analogous one in which water was the hemolytic agent, it appeared that in the first case the amount of sediment of cell wall material was less than in the second one. The breakdown of the cell wall structure and the partial dissolution of this material in a glycolipid solution becomes evident after the contents of the tubes were hand-shaken and left at room temperature for some hours. By this treatment the cell walls were destroyed. A great part of the material of the cell walls passes into the glycolipid solution while the debris remain dispersed in the medium.

From the foregoing can be deduced, that the hemolytic action of the glycolipid is due to the damage to the cell wall of the red blood cells. Even when a hypertonic solution of the glycolipid (glycolipid in normal saline) was used, so that hemolytic action cannot be ascribed to a lower osmotic pressure of the solution, hemolysis occurred. It seemed indicated to make suspensions of the cell walls of erythrocytes. A study of the effect of sodium glycolipid on isolated cell walls showed conclusively that the glycolipid readily dissolves a part of the material of the cell wall. When *e.g.* some drops of a 2 per cent solution of sodium glycolipid was added to a suspension of cell walls, an instantaneous clearing took place, whereas in a suspension of cell walls to which the same volume of water was added the turbidity remained unchanged.

It is known that the erythrocytes of warmblooded animals have a quite constant composition, not only in individuals of the same species but also in different species and moreover that the cell wall of these erythrocytes has a characteristic lipid composition. It is very likely that the glycolipid acts as a hemolytic agent like bilesalts, ether, chloroform, alkali oleates, lysolecithin and saponines which dissolve the lipids of the cell wall. The hemolytic action of those compounds indicates that the lipid fraction is of paramount importance to the cell wall structure of the red blood cells.

Summary.

The toxicity of the glycolipid of *Ps. aeruginosa* to mice has to be ascribed to the fact that it causes hemolysis by dissolving materials from the cell wall structure of the red blood cells.

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SOME REMARKS ON AUTOLYSIS OF *PSEUDOMONAS AERUGINOSA*

by

G. SIERRA and **R. ZAGT**

(Received November 13, 1959).

1. Introduction.

During our investigations on esterases it was observed that shaken cultures of *Ps. aeruginosa* in broth media became clear after five days of incubation at 37° C., while at the same time the ali-esterase activity increased (Sierra, 1957b). This observation suggests that there may be a relation between both phenomena.

The higher transparency after some days of growth may be caused by a change of the cell materials by which the cells become more transparent, or by (auto-)lysis which destroys the cells. Our attention was drawn to lysis because ali-esterase as an endocellular enzyme becomes detectable only after lysis of the cells.

Previously lysis of *Ps. aeruginosa* was considered as a spontaneous phenomenon accompanied by dissociation (Hadley, 1924, 1927). It can also be caused by bacteriophage (Dickinson, 1948; van der Ende and Don, 1952). As however in some cases the activity of phages could be excluded, it was supposed that lysis might be due to metabolic processes (Socías, 1946).

In this paper will be described the lysis of a strain of *Ps. aeruginosa*.

2. Methods.

Suspensions for electron micrographs were made as follows. Nutrient broth cultures were centrifuged at 20000 r.p.m. during 30 minutes. The sediment was washed twice with sterile water using the same time and speed of centrifuging as before. Finally a suitable suspension was made in sterile water.

Photomicrographs were taken with a normal microscope or a stereomicroscope and a Zeiss Miflex camera on Ilford "Orthotone" and "Selochrome" plates.

Culture filtrates were made by means of Zsigmondy membranes of different pore size, which were placed in Grabar filters from Schott.

The preparation of cell extracts has been described elsewhere (SIERRA, 1957b).

Suspensions of heat killed cells (h.k.c.) were prepared with cells harvested from 24 hours old broth cultures. The cells were washed and then a dense suspension in water was heated during 20 minutes at 100° C.

To detect the excreted lytic enzymes in broth cultures, the cells were removed by centrifuging. The cell free liquid was tested after different treatments:

a. the liquid as such was boiled during different length of time;
b. the liquid was concentrated from 8 to 1.5 ml by dialysing against a 15% solution of carboxymethylcellulose XT 843 (A.K.U., Arnhem, Holland) as suggested by WOLVIUS (1955) and used in the same manner by GREUELL and SIERRA (1957); this concentrate was boiled during periods of different length.

3. EXPERIMENTS AND RESULTS.

A. Properties of the strain.

The strain was found as an infection in enrichment cultures of *Mycobacterium phlei* from canalwater. The properties of the culture are in agreement with those of *Ps. aeruginosa* given by BERGEY (1948). The production of pyocyanine by our strain in (Difco-) broth was however extremely small. The dissimilation of carbohydrates was the same as described by PINGHUI LIU (1952). From the 19 amino acids tested only glycine, alanine, phenylalanine and tyrosine were suitable to induce the production of pyocyanine in a synthetic medium. In the case of valine and phenylalanine the differences with the results of BURTON (1947) perhaps may be ascribed to differences in the media. Our medium contained 0.2% K_2HPO_4, 0.2% NaCl, 0.02% KNO_3, 0.02% $MgCl_2$ and amino acids in concentrations of 0.5 to 1.0%. Since, in contrast to BURTON, we got in our synthetic medium without glycerol a fairly good production of pyocyanine, we are not convinced of the absolute necessity of

this compound for the production of the pigment (*cf.* HILLIGER, 1951).

From some experiments of ELEMA (1931) and P. LIU (1952) we also got the impression that glycerol only plays a stimulating role. The fact that there were formed greater amounts of pyocyanine in meat extract than in Difco broth must be ascribed to the greater amounts of fat present in the former medium. Hydrolysis of this fat by lipase to glycerol causes a higher production of the pigment in the meat extract. It was also found that in Difco nutrient broth and other media addition of glycerol or tri-glycerides — *e.g.* tri-butyrine, which is split by *Ps. aeruginosa* — stimulated the production of pyocyanine.

A fluorescent pigment was observed in peptone agar slant cultures by means of UV light.

We also observed a red colour in cultures in synthetic media provided with glycine or alanine, and in gelatine plus KNO_3 media provided with Difco casamino acids, proteose- or Bacto-peptone. For observing the "red pigment" one has to use alkaline solutions because in acid solutions the red configuration of pyocyanine may be misleading. In some preliminary experiments we tried to isolate this "red pigment". Cultures were made in media of casamino acids with gelatine and KNO_3 and were incubated at 37° C. during four days. Afterwards the liquid medium was saturated with $(NH_4)_2SO_4$ and extracted with 96% ethanol. The red alcoholic layer was separated. However, when we tried to remove the proteins from this alcohol-water layer by fractionated precipitation by means of 96% ethanol or by phosphowolframate, the red colour vanished. The same trouble we met if we treated in the same way silica-gel that through a collodion membrane had adsorbed the "red pigment" from a thin layer of a peptone agar culture. So we could not get solutions of the pigment pure enough for spectro-analysis.

In contrast with *Ps. fluorescens, Ps. aeruginosa* is able to form a glycolipid. This compound was isolated by JARVIS and JOHNSON (1949) from broth cultures with 3% glycerol. We isolated it from our culture in the same way (SIERRA, 1960).

The isolation and the properties of oxy-chlororaphine are described elsewhere (SIERRA and VERINGA, 1958).

We found the same suitability of various compounds for growth as has been found by ROBINSON (1932).

The colonies on peptone agar, nutrient broth agar and meat

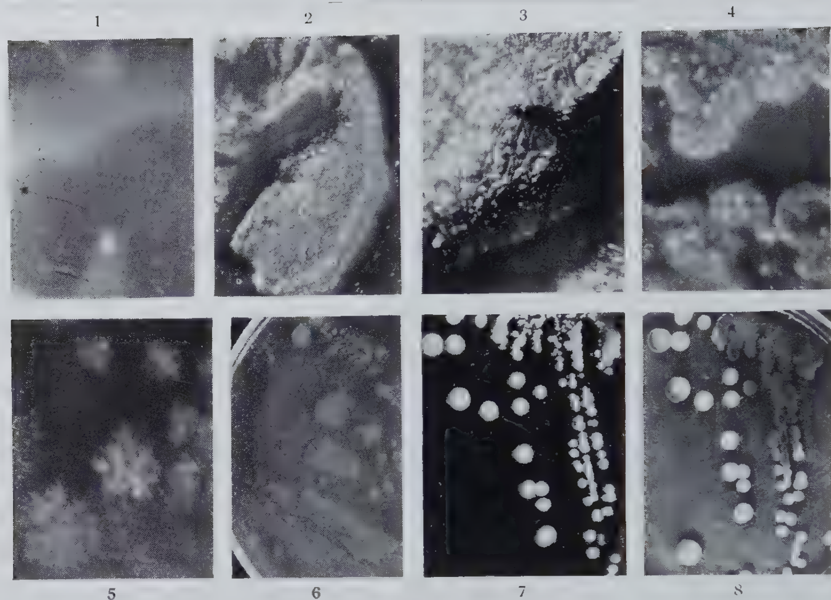

Fig. 1. Lytic zone in a colony of *Ps. aeruginosa* on peptone agar plate after 5 days of incubation, dark field; 160 ×.

Fig. 2. Lytic zone in a colony of *Ps. aeruginosa* on peptone agar under slanting rays; 160 ×.

Fig. 3. The same as fig. 2.; 250 ×.

Fig. 4. Crystals in lysed colonies of *Ps. aeruginosa* on meat extract agar after 7 days of incubation; 60 ×.

Fig. 5. The same as fig. 4; 400 ×.

Fig. 6. Lysed colonies on nutrient broth agar of *Ps. aeruginosa* after 5 days of incubation.

Fig. 7. Nutrient broth agar culture with glucose of *Ps. aeruginosa* after 5 days of incubation.

Fig. 8. Nutrient broth agar culture with glucose of *Ps. aeruginosa* after 8 days of incubation.

extract agar show an iridescent effect already observed among others by Hadley (1924, 1927), Warner (1950), and Dickinson (1952). Some areas become transparent after some time (fig. 1) and have a glittering appearance when they are illuminated by slanting rays from some specific direction (figs. 2 and 3). These areas are heavily shrivelled and folded. When observed with transmitted light, these folds might be confused with crystals, which are formed under certain conditions. On meat extract agar and on peptone agar with Tween our strain produced crystals of the same kind as described

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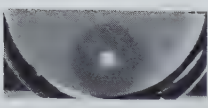

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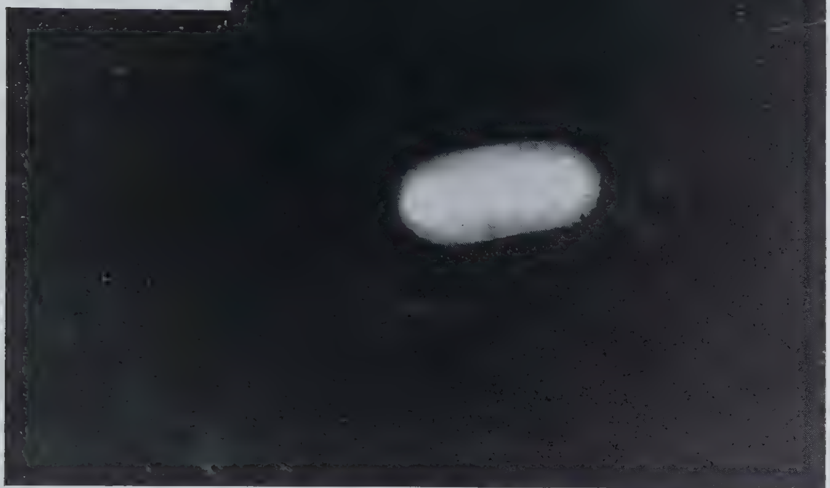

Fig. 9. Digestion of heat killed cells of *Ps. aeruginosa* by proteolytic activity present in the culture medium of a 3 days old culture.

Fig. 10. Electron micrograph of a normal cell of *Ps. aeruginosa* from a 16 hrs old nutrient broth culture; shadowed; 25000 ×.

by Lisch (1924), Pesch and Sonnenschein (1925) and Socías (1946) as shown in figs. 4 and 5. However, on mere peptone agar these crystals could never be detected although the glittering effect was present, so there seems to be no reason to suppose a relation between the "crystals of Lisch" and the iridescent phenomenon. Furthermore other lipolytic bacteria, *e.g. B. macerans*, produce the same kind of crystals on meat extract agar and on peptone agar with Tween without iridescence. It seems rather probable that they are insoluble Ca-salts of fatty acids, which are liberated by lipolysis of the lipids in both media (Sierra, 1957a).

B. The lytic phenomenon.

In the first place we investigated whether or not bacteriophages were present.

1. Electron micrographs were taken of the cultures at different stages of development. However, we could not detect any of the phages, which are so clearly visible on the pictures of van der Ende and Don (1952). Also the course of the lytic process (figs. 10 to 14)

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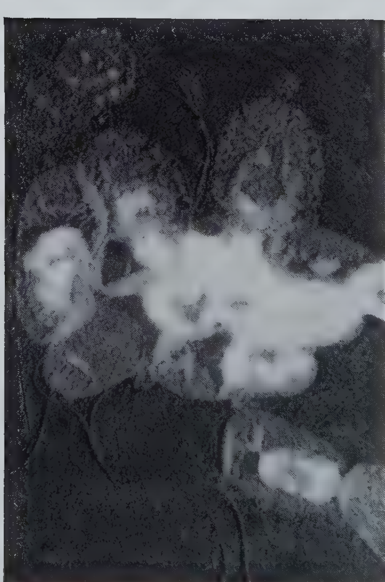

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is quite different from what might be expected from phage activities.
2. Washed cells from a 16 hours old culture were suspended in tubes with solutions of 0.85% NaCl in water, respectively in tubes with a phosphate buffer (pH 7.4). Both suspensions were kept — under toluene — at 37° C., while duplicates remained at 2° C. under such conditions that proteolytic activity might be neglected (RICHOU, 1953). The turbidity remained unchanged in all tubes during several weeks. In the cell free liquid from these tubes it was also impossible to detect ali-esterase activity with the Warburg technique.
3. Tubes with suspensions of washed young cells in 0.85% NaCl solutions were provided with some drops of culture filtrates from 5 days old autolysed cultures. For the filtration we used membranes of pore size 350 mμ as well as 35-20 mμ. The tubes were incubated at 37° C. Turbidimetric measurements after 7 and 16 hours showed no change in the turbidity of the suspensions.
4. The same kind of suspensions were then provided with some drops of cell extracts of *Ps. aeruginosa*. Here too no change in turbidity was observed.

From these experiments it may be concluded that bacteriophages are not present. Attention is called to the results in exp. 2. The method used here is often described as a suitable one to get preparations of endocellular enzymes because of the autolysis by enzymes present *in* the cells themselves (UMBREIT *et al.*, 1951, HUGO, 1954). This method failed in our case. So it seems that only in growing cultures lysis occurs.

The following experiments were carried out to observe the effect of high concentrations of some metabolic products of *Ps. aeruginosa* on growing cultures of this strain.
5. In an auxanographic experiment were investigated the influence

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Fig. 11. Electron micrograph of a lysing cell of *Ps. aeruginosa* from a 24 hrs old nutrient broth culture; shadowed; 25000 ×.

Fig. 12. Electron micrograph of lysed cells of *Ps. aeruginosa* from a 4 days old nutrient broth culture; shadowed; 25000 ×.

Fig. 13. Electron micrograph of lysed cells of *Ps. aeruginosa* from a 4 days old nutrient broth culture; shadowed; 25000 ×.

Fig. 14. Electron micrograph of cell debris from a 5 days old nutrient broth culture of *Ps. aeruginosa*; shadowed; 8750 ×.

of: *a*) pyocyanine, because of its cytolytic and bactericidal properties (HETTCHE, 1932); *b*) an emulsion of oleic acid (stabilised with gum arabic), because HETTCHE (1934) ascribed the bactericidal activity of "pyocyanase" to the fact that unsaturated fatty acids are liberated; *c*) the Na-salt of the glycolipid, because of its toxicity (JARVIS and JOHNSON, 1949) and its hemolytic effect (SIERRA, 1960).

These compounds did not inhibit the growth, so they cannot be responsible for the lytic phenomenon. Now it was observed by SIERRA (1957c) that the ali-esterase activity of washed cells depended on the presence of glucose, Tween and other compounds in the broth medium from which they are harvested. Especially the compounds, which are able to lower the concentrations of the Ca-ions in the medium, inhibit the activity of the ali-esterase of the cells harvested from such a medium.

Because of this we followed the turbidity of the cultures in different media.

6. The media were incubated with equal amounts of 24 hours old cultures. In all media the starting pH was 7.4, which remained constant during the logarithmic phase, but increased more or less afterwards. There is no connection between the autolysis and the increase of the pH. This experiment also confirmed the independence from pyocyanine of the lytic phenomenon. When, in some media, this pigment was formed, it was removed by gentle shaking with a drop of $CHCl_3$ before the optical density was measured.

The results reproduced in fig. 15 indicate that the rate of lysis is more decreased by oxalate and glucose (*cf*. fig. 6 with figs. 7 and 8) than by phosphate. Now it is a well known fact that glucose has a protein sparing effect (KENDALL, 1918; DOZIER, 1924), while oxalate and other compounds which are able to bind Ca-ions decrease the activity of proteolytic enzymes (GORINI, 1951; GORINI and FROMAGEOT, 1950). This is corroborated by GORINI's statement that Ca-ions have a stabilising effect on these enzymes.

Therefore we tested under different conditions the gelatine liquefaction with *Ps. aeruginosa* and several other bacteria. Also cell extracts of *Ps. aeruginosa* were used.

7. The experiments were carried out on plates. In order to get a good precipitation of the insoluble Ca-compounds, Na-oxalate and the phosphates were added before sterilisation. When testing the diffusibility of the enzymes through the membranes, the bacteria were inoculated directly upon these membranes, which covered the

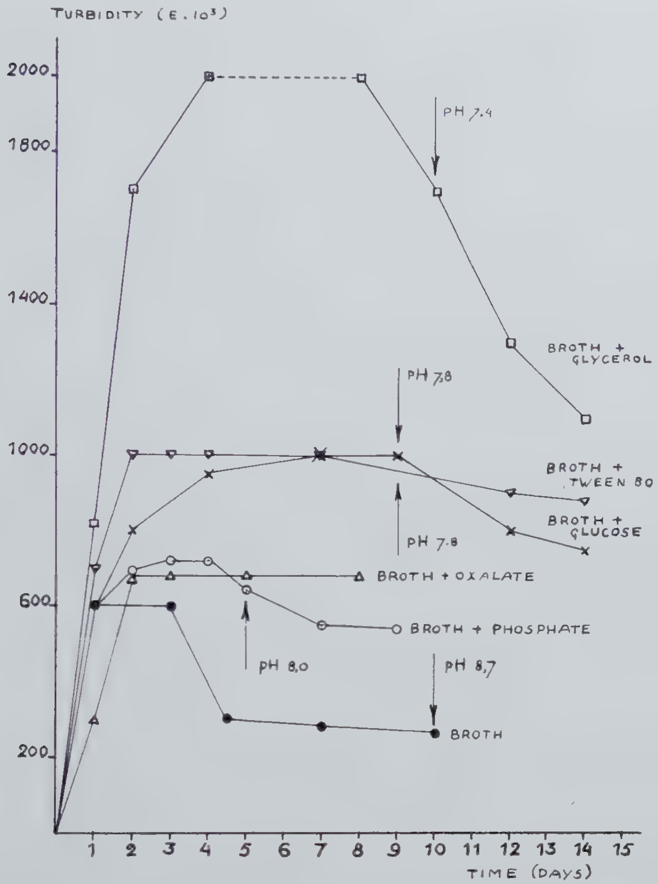

Fig.15. Influence of different compounds on the rate of autolysis of cultures of *Ps. aeruginosa* in nutrient broth.

gelatine plates. In all cases the incubation temperature was 22° C. The difference in proteolytic activity was recorded by means of the length of time whereafter liquefied gelatine could be noted around the colonies. The liquid cell extract was collected on pieces of filter paper which were brought upon the gelatine plates. In order to get some impression of the enzymatic activity of these extracts paper disks provided with a small amount thereof were brought upon peptone agar plates with Tween (SIERRA, 1957a). A high lipolytic activity was detected.

TABLE 1.

Inhibition of the activity of gelatine liquefying enzymes by some compounds and the diffusibility of these enzymes through membranes.

bacteria	Inhibition			permeability of the membranes		
	K_2HPO_4 1%	glucose[1]) 1%	Na-oxalate 1%	cellophane	Zsigmondy 20-35 $m\mu$	Zsigmondy 350 $m\mu$
Ps. aeruginosa	+	+++	+++	—	+	++
Bac. subtilis 9	+	±	++	—	+	++
Bac. pumilus 24	+	±	++	—	+	++
Bac. megaterium 46	+	+	++	—	+	++
Micrococcus pyogenes var. *aureus*	+	±	++	—	+	++

E. coli and cell extracts of *Ps. aeruginosa* did not liquefy gelatine at all.

[1]) In this experiment the medium was buffered with KH_2PO_4 and Na_2HPO_4.

Concerning the effect of Ca-binding compounds, our results (table 1) are in agreement with those of GORINI. Furthermore, it does not seem to make any difference if we use filtered or centrifuged cultures to investigate the proteolytic enzymes, because these pass through Zsigmondy membranes. Proteolytic activity has not been found among the endocellular material in the cell extracts of *Ps. aeruginosa*, but as the proteolytic enzymes excreted into the medium are influenced in the same way as the lytic agent, it might be supposed that proteolytic enzymes of *Ps. aeruginosa* are able to digest their own cells. However, exp. 3 shows that washed young cells are not attacked by proteolytic enzymes present in the filtrate of an autolysed culture.

In order to get more information of this digestion we made the following experiments.

8. Petri dishes were provided with a heavy suspension of washed young cells in peptone agar at 45° C. After being chilled to 3° C. the plates were covered with a very thin layer of washed agar. Some disks of filter paper with small amounts of concentrated autolysate were brought upon the plates. After incubation at 37° C. no clear zones could be noted around these disks which would have indicated the occurrence of digestion.

9. If living cells are not attacked, they must be able to grow in the

medium of a heavy autolysed culture after the original bacteria have been removed by centrifuging or filtration through Zsigmondy membranes. This filtrate and a normal broth medium were inoculated with the same amounts of cells harvested from a 24 hours' culture. In both media growth occurred after incubation at 37° C. during 24 hours. Although the growth in the filtrate was slower at the start — perhaps because the initial necessary substrates had been consumed before the filtration — the turbidity in both media was the same after 48 hours.

So the viable cells of *Ps. aeruginosa* are not attacked by their own excreted proteolytic enzymes. Dead cells, however, might be destroyed.

10. In order to establish this, dead, heat killed cells (h.k.c.) were brought into contact with the proteolytic enzymes under different conditions.

a. The h.k.c. were suspended in peptone agar, to which in some cases chemicals had been added. On these agar plates several species of bacteria were streaked and incubated.

b. To test the diffusibility through cellophane the cultivation of the bacteria took place directly on the cellophane membrane which covered the agar.

c. In order to test the culture liquid (fig. 9) or the cell extract, small volumes thereof were brought on disks of filter paper, which were placed upon the agar.

The results in table 2 show, that *Ps. aeruginosa* excretes a thermolabile enzyme, that is able to digest the h.k.c. of *Ps. aeruginosa* itself and several other kinds of bacteria. The activity of these enzymes is influenced by glucose, oxalates and phosphates in the same way as the proteolytic enzymes which liquefy gelatine, and as the enzymes responsible for the autolysis in broth cultures. None of them can pass through cellophane, so these phenomena seem to be caused by the same proteolytic enzymes. It is remarkable that cell extracts of *Ps. aeruginosa* digested neither gelatine nor h.k.c. It seems therefore that the autolytic processes in cultures of *Ps. aeruginosa* have to be ascribed to the destruction of dead cells by the excreted proteolytic enzymes.

4. Discussion.

Very little is known about the red colour of *Ps. aeruginosa* (*cf.* Meader *et al.*, 1925). It is mostly found in older cultures of *Ps.*

TABLE 2.

Digestion of heat killed cells by proteolytic activities of bacteria.

bacteria or preparation tested	h.k.c. of the following species	chemicals added	digestion after:		
			24 hrs	48 hrs	72 hrs
Ps. aeruginosa	*Ps. aeruginosa*	none	+	++	+++
,,	idem, under cellophane	,,	—	—	—
,,	*Micrococcus pyogenes* var. *aureus*	,,	+	++	+++
,,	idem, under cellophane	,,	—	—	—
,,	*Bac. subtilis* 9	,,	+	++	+++
,,	idem, under cellophane	,,	—	—	—
Micrococcus pyogenes var. *aureus*	*Micrococcus pyogenes* var. *aureus*	,,	+	++	+++
,,	*Ps. aeruginosa*	,,	—	—	—
Bac. subtilis 9	,,	,,	—	±	+
Bac. pumilus 24	,,	,,	+	++	+++
Bac. megaterium 46	,,	,,	+	++	+++
Bac. cereus	,,	,,	+	++	+++
E. coli	,,	,,	—	—	—
Ps. fluorescens non-liquefaciens	,,	,,	—	—	—
Ps. aeruginosa	,,	0.1% glucose	+	++	+++
,,	,,	1.0% ,,	—	±	+
,,	,,	1.0% K_2HPO_4	—	±	+
,,	,,	1.0% NaH_2PO_4	—	—	±
,,	,,	1.0% Na-oxalate	—	—	—
Ps. aeruginosa (cell extract)	,,	none	—	—	—
Ps. aeruginosa (autolysate)	,,	,,	+	+	+
idem (concentrated)	,,	,,	++	++	++
idem (concentrated and boiled 5 min.)	,,	,,	±	±	±
idem (concentrated and boiled 15 min.)	,,	,,	—	—	—

aeruginosa. GESSARD (1917) *e.g.* observed this colour in broth cultures after 5 days of incubation. As the length of this period coincides with the time the autolysis becomes clearly visible, there may be a relation between these phenomena.

The metallic glittering effect is ascribed to lysis (SOCÍAS, 1946; WARNER, 1950; DICKINSON, 1952). In agreement with SOCÍAS we could exclude the activity of phages. Moreover, some preliminary experiments showed that glucose and oxalate influence the transparency, the shrivelling and the metallic glittering outlook of the colonies in the same way as the autolysis.

The fact, that the inhibitory effect upon the lysis increases when the insolubility of the Ca-compounds becomes greater (see table 2), demonstrates the fundamental role of the Ca-ions in the lytic phenomenon. The retarding effect on autolysis by Tween 80 may be explained with an analogous argument. It is known that Tween 80 is hydrolysed by the lipase of *Ps. aeruginosa* (SIERRA 1957a) so that the oleic acid liberated is able to combine with Ca-ions to a rather insoluble salt giving rise to a decrease of the rate of lysis.

The fact that the autolysis of *Ps. aeruginosa* in submerged cultures has to be ascribed to the digestion of the dead cells by excreted proteolytic enzymes, is based upon the following results:

a. Autolysis in submerged cultures, digestion of h.k.c., and liquefaction of gelatine are influenced by the same factors.

b. Living cells are not attacked by their excreted proteolytic enzymes.

c. Washed cell suspensions do not autolyse when osmosis is prevented.

d. Cell extracts show neither any proteolytic activity, nor a liquefaction of gelatine, or digestion of h.k.c.

It is remarkable that *Micrococcus pyogenes* var. *aureus* (WELSCH, 1949) and other staphylococci (GRATIA and RHODES, 1924), *Streptomyces albus* (WELSCH, 1937) and some other *Streptomyces* species (WAKSMAN, 1950), *B. cereus* (NORRIS, 1957) and *B. subtilis* (NOMURA and HOSODA, 1956) autolyse in submerged cultures, while at the same time these species are able to digest gelatine as well as h.k.c. of their own and several other species. These facts support our first observation showing a relation between the three phenomena, although the different influence on the lysis by glucose and phosphate in the case of *Micrococcus pyogenes* var. *aureus* (WELSCH, 1949) seems to be an exception.

In connection with this it must be noted that the excreted proteolytic enzymes have some specificity with respect to the kind of h.k.c. they digest. Furthermore, the enzymes of *Ps. aeruginosa* do not seem to be able to digest flagella (figs. 12 and 13), although flagella have a protein constitution (keratine — epidermine — fibrinogene; Weibull, 1948, 1950).

It has been shown that the supernatant of lysed cultures of *Micrococcus pyogenes* (Welsch, 1949), *B. cereus* (Greenberg and Halvorson, 1955), *B. subtilis* (Nomura and Hosoda, 1956) and *Cl. botulinum* (Boroff, 1955) digest young living cells of their respective strains. This may be caused by the combined action of the excreted proteolytic enzymes and bactericidal substances (*B. cereus*, Norris, 1957; *Streptomyces albus*, Welsch, 1937) or by cell-wall attacking substances (*B. subtilis*, Nomura and Hosoda, 1956). The proteolytic enzymes themselves are active only upon the damaged cells.

The work of Greenberg and Halvorson (1955) and Norris (1957) on *B. cereus* gives important support to our results with *Ps. aeruginosa*. In fact in both cases the presence of endocellular proteolytic enzymes could be excluded as the cell extracts did not show (proteo-)lytic activity. The activity of endocellular material of *Micrococcus pyogenes* on h.k.c. is nihil (Welsch and Salmon, 1950) but has not yet been tried on living cells.

On account of these facts it seems worthwhile to investigate the autolytic process from the point of view that the autolysis of gelatine-liquefying species may be caused by earlier excreted proteolytic enzymes, which are active upon cells damaged in some way. A consequence of this assumption would be that it is not any more allowed to ascribe automatically the autolysis of cultures, which have stopped to grow, to the activity of endocellular enzymes (*cf.* Waksman, 1950).

A rather weighty support to our results is provided by the fact that all species mentioned above were gelatine liquefying ones, while they do not contain endocellular proteolytic enzymes. On the other hand, several gelatine non-liquefying microbes as yeasts (Joslyn, 1955) and pneumococci (Dubos, 1937) have endocellular proteolytic enzymes able to digest proteins of h.k.c. of their own species.

Summary.

Several observations on the autolysis in cultures of *Ps. aeruginosa* are described. Autolysis appears to be caused by thermolabile and non-dialysable substances, which are identified as excreted proteolytic enzymes. These enzymes only digest dead cells.

Acknowledgement.

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(Aus dem Institut für Tropische Hygiene und Geographische Pathologie, Abteilung des Königlichen Instituts für die Tropen, Amsterdam).

DIE ZÜCHTUNG DES GELBFIEBERVIRUS 17D IM ASCITES DES KREBS-2-CARCINOMS

von

W. A. COLLIER und **M. DE WIT**

(Empfangen 25. August 1959).

Nachdem zuerst DAELS (1910) auf den Antagonismus zwischen Carcinom-, Spirochaeten- und Trypanosomeninfection aufmerksam gemacht hatte [vergl. auch CERUSIA (1928)], waren LEVADITI und NICOLAU (1923) die ersten, die den Antagonismus zwischen einem Virus und Neoplasmen näher untersuchten. Es folgten die Untersuchungen von RIVERS und PEARCE (1925) und PEARCE und RIVERS (1927), und heutigentags liegen bereits zahlreiche Untersuchungen vor, von denen nur die von ATANASIU und LÉPINE (1959), BAUER und GRAND (1954), BIERMAN, HAMMON, EDDIE, MEYER und SHIMKIN (1950), GINDER und FRIEDEWALD (1951, 1952), HALLAUER (1931), KOPROWSKI und NORTON (1950), KOPROWSKI, LOWE und KOPROWSKA (1957), KUWATA (1951), LEVADITI und HABER (1936, 1937), LEVADITI und SCHOEN (1936), LOWE und SHARPLESS (1954), MOORE (1949, 1951, 1952, 1953, 1954), MOORE und O'CONNOR (1950), SHARPLESS, DAVIES und COX (1950), SOUTHAM, BRONSTEIN und WEBER (1951), SOUTHAM und EPSTEIN (1953), SOUTHAM und MOORE (1951, 1952), TOOLAN und MOORE (1952), TURNER und MULLIKAN (1947, 1950) und TURNER, MULLIKAN und KRITZLER (1948) genannt seien.

Von den Versuchen, verschiedene Virusarten in Ascites-Tumoren zu züchten, seien nur die von FLANAGAN und COLTER (1955), KOPROWSKA und KOPROWSKI (1952, 1953), KOPROWSKI, KOPROWSKA und LOWE (1953), KOPROWSKI und LOWE (1953, 1954), LOWE, KOPROWSKI und COX (1953) und LUTIKOVA (1957) genannt.

Von besonderem Interesse sind aber die Versuche Gelbfiebervirus in Tumoren zu züchten. Als erste arbeiteten FINDLAY und

MacCallum (1937) mit dem soliden Mäusecarcinom „63" und einem Mäusesarcom. Im Carcinom konnte Virus 12-14 Tage nachgewiesen werden, nicht aber im Sarcom. Passagen in Symbioseform waren aber nur einmal bis zur 5. Passage möglich, da das Gelbfiebervirus Necrose der Tumorcellen verursachte, die infolgedessen bei der Verimpfung auf neue Mäuse nicht weiter zur Entwicklung gebracht werden konnten. Bei den Passagen wurde daher das Material in neue bereits wachsende Tumoren gebracht. Auf diese Weise wurde der neurotrope Dakarstamm, der viscerotrope (pantrope) Dakarstamm und der Vaccinstamm 17 E 50–60 Passagen weitergezüchtet.

In den Versuchen von Koprowski und Norton (1950) glückte es nicht, den neurotropen Dakarstamm im Mäusesarcom 180 (Crockersarcom) weiter zu züchten. Ebenso konnte Koprowski (1956) im Ehrlichschen Ascitescarcinom keine Virusvermehrung feststellen.

In den hier mitgeteilten Versuchen sollte festgestellt werden, ob das Gelbfieber-Vaccinvirus 17D in dem in Ascitesform wachsenden Krebs-2-Carcinom weiter gezüchtet werden konnte und ob es hierbei zu einer Abschwächung der Mäusevirulenz kommt. Ferner sollte festgestellt werden, ob auch der Ascites-Carcinomstamm in seiner Virulenz beeinflusst wurde. Die Virulenz wurde in beiden Fällen an Hand von Virulenzcurven nach Collier, de Roever-Bonnet und Hoekstra (1959) bestimmt, nachdem Collier und de Wit (1960) die Anwendung dieser Methodik bei der Bestimmung von Virulenz von Ascitestumoren durch intracerebrale und durch intraperitoneale Titration empfohlen hatten.

Technik.

Die Versuche wurden mit dem in Ascitesform wachsenden Stamm des Krebs-2-Carcinoms und dem Gelbfieber-Vaccinestamm 17 D durchgeführt. Die Passagereihe wurde damit begonnen, dass weisse Mäuse mit 0,2 ml unverdünntem Ascites und 0,2 ml Gelbfiebervirus 17D in der Verdünnung von $^{1}/_{100}$ gleichzeitig intraperitoneal inficiert wurden.

Nach 7–8 Tagen wurde von den inzwischen durch die Ascitesbildung sehr dick gewordenen Mäusen 1–2 ml Ascites abgenommen und in der Menge von $\pm$ 0,2 ml auf eine weitere Serie weisser Mäuse übertragen. Dies wurde bisher länger als ein Jahr fortgesetzt.

In verschiedenen Abständen wurde die Virulenz des im Ascites

weitergezüchteten Gelbfiebervirus bestimmt. Zu diesem Zweck wurde zu verschiedenen Zeiten nach der Infection Ascites abgenommen und 10 Minuten lang bei 5000 Umdrehungen zentrifugiert. Von dieser völlig klaren Flüssigkeit wurden weitere Verdünnungen gemacht und intracerebral in der Menge von 0,02 ml bei Serien weisser Mäuse eingespritzt.

Um eine mögliche Beeinflussung des Ascitescarcinoms durch das Gelbfiebervirus festzustellen, wurde zu verschiedenen Zeiten nach der Infection Ascites in fallenden Verdünnungen sowohl intracerebral als auch intraperitoneal auf Serien weisser Mäuse verimpft. Zum Vergleich wurden ebenso alte Proben von Ascites ohne Gelbfiebervirus in genau der gleichen Weise untersucht. Von diesen Versuchen wurden die Virulenzcurven ausgerechnet.

Die Bestimmung der immunisierenden Kraft des im Ascites sich entwickelnden Gelbfiebervirus erfolgte derart, dass zwei Serien weisser Mäuse intraperitoneal mit Gelbfiebervirus von Ascitespassagen zweimal immunisiert und danach mit dem Stamm 17D intracerebral nachinficiert wurden. Von den erhaltenen Ergebnissen wurden Virulenzcurven berechnet.

Zur Erhöhung der stark abgeschwächten Virulenz für die Maus wurde das Ascitesvirus intracerebral durch weisse Mäuse weitergezüchtet, nachdem die Ascitescellen durch Centrifugieren entfernt worden waren. Von der 5. und 13. Gehirnpassage wurden Virulenzcurven ausgearbeitet.

Besprechung der Ergebnisse.

In Tabelle 1 findet sich eine Uebersicht über die LD_{50} des Gelbfiebervirus aus verschiedenen Ascites-Passagen nach verschiedenen Intervallen zwischen Infection und Virus-Titrierung. Am 17. und am 19. Tage nach der Infection ist kein Virus mehr nachweisbar. Mit zwei Ausnahmen liegt die LD_{50} in der Regel unter $^{1}/_{100}$, während sie für den 17D-Stamm in der Regel erheblich höher liegt. Es ist deutlich, dass die Virulenz des Stammes 17D für die weisse Maus durch die Passagen in Ascites vermindert wird.

Im Verlauf der Passagen bekommt man deutlich den Eindruck, dass die Mäuse, welche mit Ascitescarcinom + Gelbfiebervirus inficiert sind, etwas länger am Leben bleiben als die nur mit Ascitescarcinom inficierten Tiere. Um dies exact festzustellen, wurden mit dem Ascites verschiedenen Alters intracerebrale Virulenzbestim-

TABELLE 1.

Titrationen des Gelbfiebervirus von Ascites-Gelbfieber-Passagen (10 Minuten bei 5000 ×) bei Mäusen: Dosis 0,02 ml intracerebral.

Passage:	Alter des Ascites in Tagen	Titer: LD_{50}
7	7	$^1/_{68}$
	7	$^1/_{1000}$
	7	$^1/_{2150}$
8	11	$^1/_{32}$
	11	$^1/_{48}$
	11	$^1/_{67}$
18	10	$^1/_{40}$
	10	$^1/_{150}$
	17	0
22	12	$^1/_{3}$
23	4	$^1/_{32}$
	5	$^1/_{56}$
	9	$^1/_{2,4}$
24	4	$^1/_{40}$
	6	$^1/_{32}$
	8	$^1/_{25}$
	19	0
25	6	$^1/_{32}$
38	4	$^1/_{18}$
	4	$^1/_{32}$
	4	$^1/_{32}$
	4	$^1/_{56}$
39	7	$^1/_{32}$
	7	$^1/_{56}$
	7	$^1/_{320}$

mungen durchgeführt. In den Abbildungen 1–6 sind von diesen Versuchen die Virulenzcurven dargestellt. Vergleicht man die Virulenzcurven des Carcinomascites mit und ohne Gelbfiebervirus miteinander, so zeigt sich in jedem Fall, dass dieses die Virulenz des

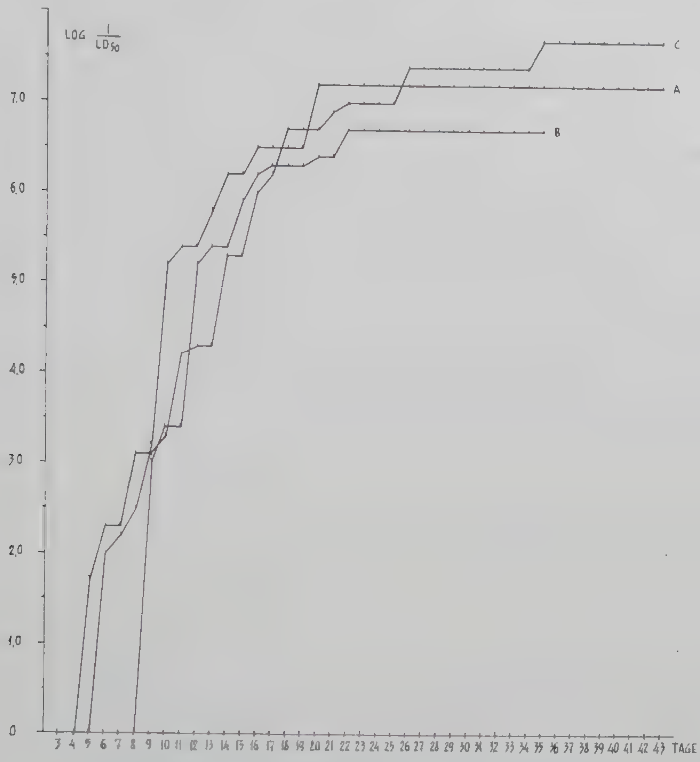

Fig. 1. Intracerebrale Infection mit 0,02 ml. Virulenzcurven von
A = 5 Tage altem Ascites mit Gelbfiebervirus
B = 5 Tage altem Ascites mit Gelbfiebervirus
C = 5 Tage altem normalen Ascites.

Ascitescarcinoms wenn auch nicht sehr stark, so doch deutlich herabsetzt.

Vergleichsweise wurden auch Virulenzbestimmungen des Ascitescarcinoms bei intraperitonealer Infection durchgeführt. Die sich hierbei ergebenden Virulenzcurven sind in Abbildungen 7–9 wiedergegeben. Bei 12 Tagen altem und 16 bzw. 18 Tage altem Ascites ergibt sich ebenso wie bei intracerebraler Virulenzbestimmung, dass der mit Gelbfiebervirus inficierte Ascites etwas weniger virulent ist als der reine Ascites. Bei 37 bzw. 36 Tage altem Ascites zeigt sich zuerst während des Verlaufes deutlich die höhere Virulenz des reinen Ascites, doch am Ende des Versuches haben sowohl der virusinficierte als auch der reine Ascites die gleichen Werte erreicht.

Zusammenfassend lässt sich also feststellen, dass durch die In-

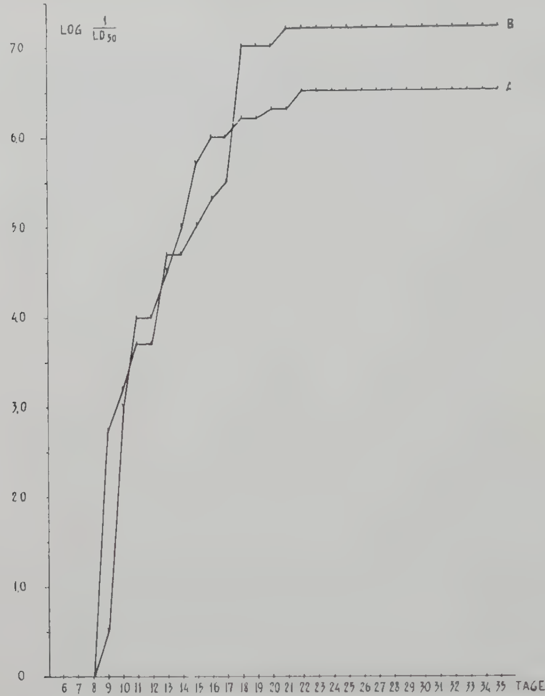

Fig. 2. Intracerebrale Infection mit 0,02 ml. Virulenzcurven von
A = 10 Tage altem Ascites mit Gelbfiebervirus
B = 10 Tage altem normalen Ascites.

fection mit dem Gelbfiebervirusstamm 17D die Virulenz des Ascitescarcinoms wenn auch nicht viel, so doch eindeutig vermindert wird.

Aus Tabelle 1 war zu ersehen, dass die Virulenz des Virus 17D vermindert war. Um festzustellen, ob die immunisierende Kraft dieses abgeschwächten Virus noch erhalten war, wurden zwei Serien weisser Mäuse intraperitoneal immunisiert, wobei jedesmal durch intracerebrale Titrierung des zur Immunisierung benutzten Ascites-Gelbfiebervirus (nach Abzentrifugieren der Ascitescellen) die LD_{50} bestimmt wurde. Die Serie A wurde immunisiert durch 0,25 ml centrifugierten Ascites mit $LD_{50} = {}^1/_{32}$ und danach durch noch einmal 0,15 ml centrifugierten Ascites von der gleichen Virulenz. Die Serie B wurde immunisiert durch 0,1 ml Ascites von $LD_{50} = {}^1/_{25}$ und danach 0,15 ml Ascites von $LD_{50} = {}^1/_{32}$. Die beiden Serien wurden in Gruppen verteilt, und zusammen mit Normalmäusen wurden die Tiere mit der Dosis ${}^1/_{10}$ bis ${}^1/_{100\,000}$ des Virus 17D inficiert.

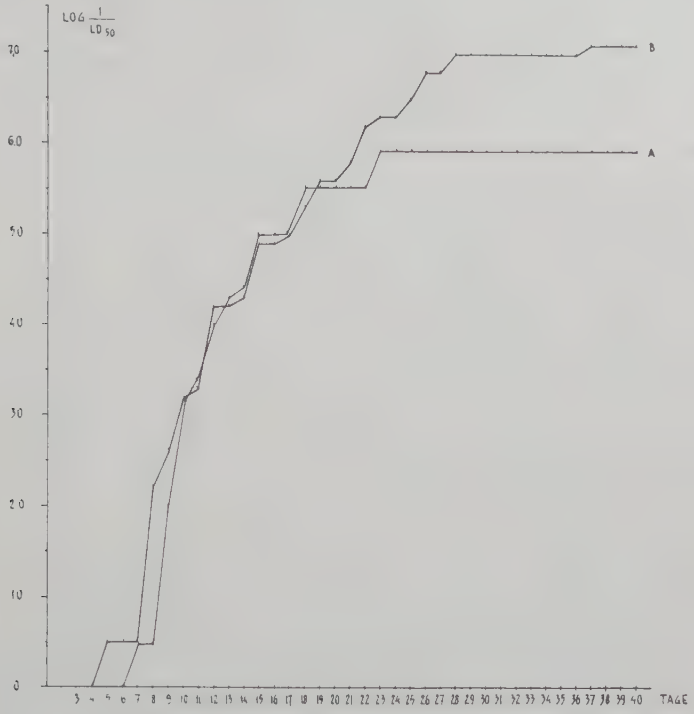

Fig. 3. Intracerebrale Infection mit 0,02 ml. Virulenzcurven von
A = 12 Tage altem Ascites mit Gelbfiebervirus
B = 12 Tage altem normalen Ascites.

Aus den Ergebnissen wurden die Virulenzcurven berechnet und in Abbildung 10 zusammengestellt. Es zeigt sich hier, dass durch die intraperitoneale Immunisierung eine sehr starke Immunität erzielt wird, ungefähr gegen die 1000-fache LD_{50}.

Um zu untersuchen, ob die zurückgelaufene Virulenz des im Carcinomascites gezüchteten Gelbfiebervirus 17D wieder zu steigern war, wurden eine Reihe von intracerebralen Passagen bei Mäusen durchgeführt. Von der 5. und von der 13. Gehirnpassage wurden Virulenzcurven ausgearbeitet und mit einer solchen verglichen, die von 4 Tage altem Virus direct aus Ascites gewonnen worden war. In Abbildung 11 sind diese drei Curven miteinander verglichen. Es zeigt sich hier deutlich, dass bereits nach 5 Gehirnpassagen eine Virulenzsteigerung erzielt werden konnte, die fast das 1000-fache des 4 Tage alten Virus aus dem Ascites ausmachte. Die Titrierung nach 13 Gehirnpassagen ergab nur um Weniges höhere Werte.

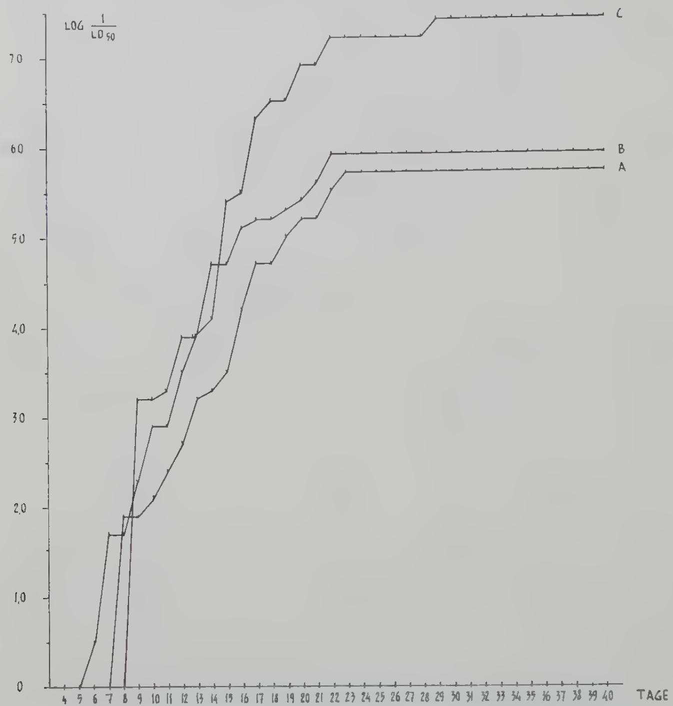

Fig. 4. Intracerebrale Infection mit 0,02 ml. Virulenzcurven von
A = 19 Tage altem Ascites mit Gelbfiebervirus
B = 18 Tage altem normalen Ascites
C = 19 Tage altem normalen Ascites.

Discussion.

Aus den hier mitgeteilten Versuchen ergibt sich zunächst, dass es möglich ist, das Gelbfiebervirus 17D praktisch unbegrenzt in dem in Ascitesform wachsenden Krebs-2-Carcinom weiterzuzüchten. Dies war früher schon Findlay und MacCallum (1937) im Mäusecarcinom „63" gelungen. In Ehrlichschen Ascitescarcinom vermehrt sich Gelbfiebervirus nach Koprowski (1956) dagegen nicht.

Gegenüber den Versuchen von Findlay und MacCallum (1937) zeigen sich aber bei den hier mitgeteilten Versuchen prinzipielle Unterschiede. Es war den genannten Autoren nich möglich, fortlaufende Passagen über die 5. Passage durchzuführen, da das Gelbfiebervirus Necrose der Tumorcellen verursachte, sodass bei der Verimpfung auf neue Mäuse das Carcinom nicht weiter zur Ent-

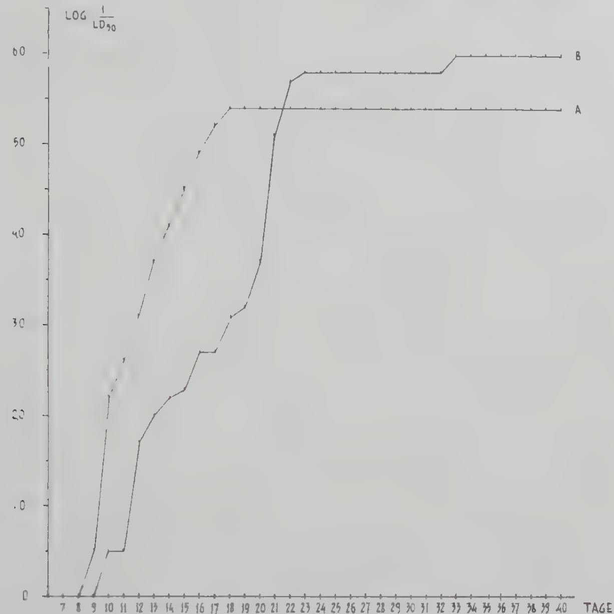

Fig. 5. Intracerebrale Infection mit 0,02 ml. Virulenzcurven von
A = 37 Tage altem Ascites mit Gelbfiebervirus
B = 36 Tage altem normalen Ascites.

wicklung gebracht werden konnte. Sie mussten daher das Virusmaterial stets in bereits wachsende, neue Tumoren bringen. In den hier geschilderten Versuchen war es aber möglich, ohne weiteres virushaltigen Ascites passageweise unbegrenzt weiterzuführen.

Ein weiterer Unterschied mit den früheren Versuchen von FINDLAY und MACCALLUM (1937) ist darin zu finden, dass die beiden neurotropen Stämme der genannten Autoren nach 50–60 Passagen in ihrer Pathogenität unverändert blieben. Sie benutzten neben dem Französischen Stamm (Dakarstamm) den Stamm 17E. Es ist nicht wahrscheinlich, dass der Stamm 17E prinzipiell stark von dem hier benutzten Stamm 17D abweicht, der während der Ascites-Passagen stark an Virulenz abnahm. Ob die Virulenz für die weisse Maus vollkommen schwinden kann, wenn die Ascitespassagen fortgesetzt werden, muss noch abgewartet werden.

Die ursprüngliche Virulenz kann durch Gehirnpassagen nicht nur sehr schnell wieder hergestellt, sondern anscheinend sogar gesteigert werden. Eine schnelle Virulenz-Zunahme durch Gehirnpassagen durch die weisse Maus sahen auch THEILER (1951) beim

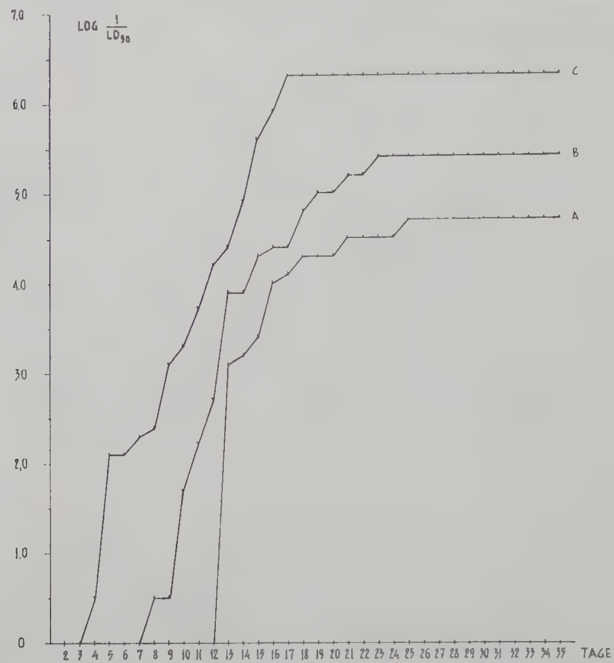

Fig. 6. Intracerebrale Infection mit 0,02 ml. Virulenzcurven von
A = 46 Tage altem Ascites mit Gelbfiebervirus
B = 43 Tage altem Ascites mit Gelbfiebervirus
C = 40 Tage altem normalen Ascites.

viscerotropen Asibistamm und THEILER (1951) und COLLIER, DE ROEVER-BONNET und HOEKSTRA (1959) beim Stamm 17D, der ja auch vom Asibistamm abgeleitet ist.

Trotz des Virulenzverlustes nach den monatelangen Passagen durch Carcinom-Ascites ist das modificierte Virus 17D doch noch imstande, nach intraperitonealer Einverleibung ausgezeichnet gegen das Virus 17D, so wie es als Vaccin gebraucht wird, zu immunisieren. Die antigenen Eigenschaften sind also durch die Ascitespassagen nicht verloren gegangen.

Die Virulenz des Ascites-Carcinoms wird durch die Meng-Infection mit dem Gelbfiebervirus leicht vermindert. Dies wird deutlich nicht nur bei intraperitonealer, sondern auch bei intracerebraler Titration, die sich in den Versuchen von COLLIER und DE WIT (1960) als besonders empfindlich herausgestellt hat. Diese Abschwächung der Virulenz ist aber nur sehr gering und keineswegs gleich zu setzen mit der hochgradigen Nekrotisierung des Carcinoms

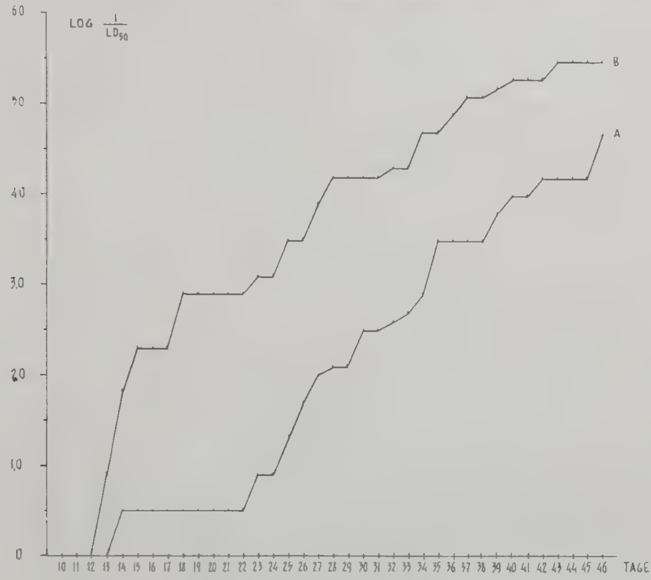

Fig. 7. Intraperitoneale Infection mit 0,2 ml. Virulenzcurven von
A = 12 Tage altem Ascites mit Gelbfiebervirus
B = 12 Tage altem normalen Ascites.

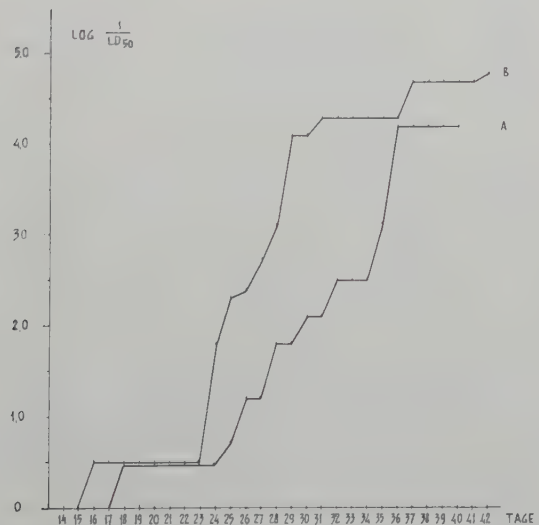

Fig. 8. Intraperitoneale Infection mit 0,2 ml. Virulenzcurven von
A = 16 Tage altem Ascites mit Gelbfiebervirus
B = 18 Tage altem normalen Ascites.

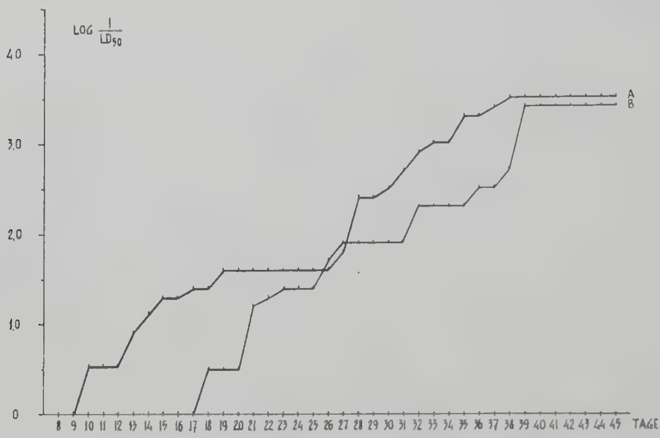

Fig. 9. Intraperitoneale Infection mit 0,2 ml. Virulenzcurven von
A = 37 Tage altem Ascites mit Gelbfiebervirus
B = 36 Tage altem normalen Ascites.

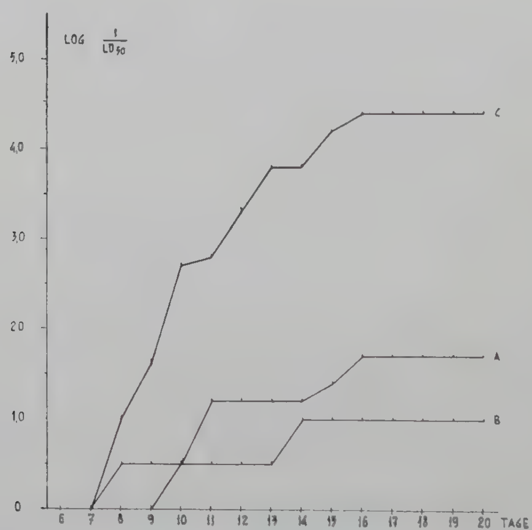

Fig. 10. Virulenzcurven nach Immunisierung mit Ascites-Gelbfiebervirus gegen den Vaccinstamm 17 D.

A = Immunisierung intraperitoneal 1 × 0,25 ml $^1/_1$ (Virulenz LD_{50} = = $^1/_{32}$) und 1 × 0,15 ml $^1/_1$ (Virulenz LD_{50} = $^1/_{32}$).

B = Immunisierung intraperitoneal 1 × 0,1 ml $^1/_1$ (Virulenz LD_{50} = = $^1/_{25}$) und 1 × 0,15 ml $^1/_1$ (Virulenz LD_{50} = $^1/_{32}$).

C = Controllversuch mit normalen Mäusen.

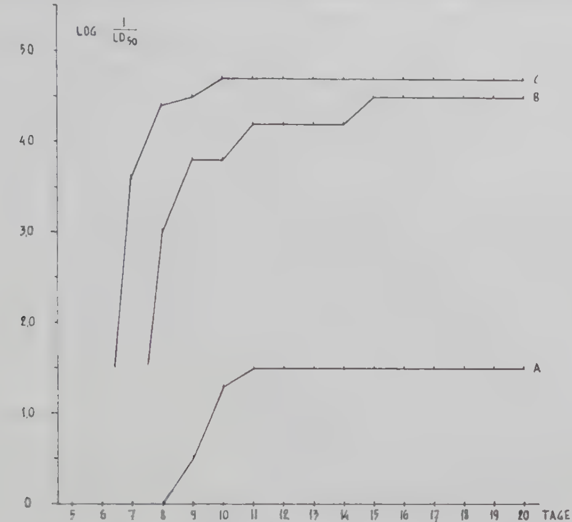

Fig. 11. Steigerung der Virulenz des Ascites-Gelbfiebervirus durch Gehirnpassagen. Virulenzcurven
A = von 4 Tage altem Gelbfieberascites
B = nach 5 Gehirnpassagen
C = nach 13 Gehirnpassagen.

„63", das in den Versuchen von FINDLAY und MACCALLUM (1937) infolgedessen nicht mehr weiter verimpft werden konnte.

Für die Titrationen des Gelbfiebervirus und für die intraperitoneale Immunisierung wurde der Gelbfieber-Ascites erst 10 Minuten lang mit 5000 Umdrehungen centrifugiert. Die überstehende Flüssigkeit erwies sich dann in der Regel als cellfrei und erzeugte in der Regel nach intraperitonealer oder intracerebraler Verimpfung keinen Carcinomascites oder solide Tumoren. Nur in einigen Fällen war durch das Centrifugieren die Carcinom-Komponente nicht völlig entfernt worden, sodass es in der Bauchhöhle bzw. im Schädel zur Ascites- bzw. Tumorbildung kam. Wenn das Material intracerebral verimpft wurde, war es stets möglich, zwischen Infection mit Gelbfiebervirus und mit KREBS-2-Carcinom zu unterscheiden, da der klinisch Verlauf sehr deutlich voneinander abweicht, und die typische Gelbfieberlähmung durch Tumorentwicklung nicht imitiert wird.

Zusammenfassung

Es ist möglich, das Gelbfieber-Vaccinvirus 17D praktisch unbe-

grenzt in dem in Ascitesform wachsenden Krebs-2-Carcinom zu züchten.

Das Gelbfiebervirus zeigt deutlich eine stark verminderte Virulenz für die Maus, die aber bei Gehirnpassagen sehr schnell wieder zurückkehrt. Die immunisierende Kraft des Gelbfiebervirus aus dem Ascites ist anscheinend vollkommen unverändert geblieben.

Der Ascites-Carcinomstamm zeigt in seiner Verbindung mit dem Gelbfiebervirus ebenfalls eine deutliche, wenn auch nur geringe Abschwächung seiner Virulenz bei intracerebraler und auch bei intraperitonealer Titrierung.

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(National Institute of Public Health, Utrecht, The Netherlands).

A NEW *SALMONELLA* TYPE (*S. LEEUWARDEN*)

by

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In December 1958 a culture was isolated by A. B. WIELENGA (Regional Laboratory of Public Health, Leeuwarden) from the stool of a five month old baby, who was affected with enteritis.

The strain possessed the cultural and biochemical characteristics typical of the genus *Salmonella*: rapid fermentation of glucose (with gas), mannitol, dulcitol, inositol, arabinose, rhamnose, trehalose, xylose and no fermentation of adonitol, lactose, salicin, sucrose; the organism failed to produce indol, was methylred positive and Voges-Proskauer negative; hydrogen sulfide was produced, but the bacteria failed to hydrolyse urea, to liquefy gelatin or to grow in KCN medium; d-, l-, and i-tartrate and mucate were utilized in 2 days.

On serological examination the organism was agglutinated by *S. aberdeen* O (11) serum.

The H-antigens were diphasic. Phase 1 was agglutinated to titre by *S. paratyphi* B, phase 1 (b) serum and phase 2 was agglutinated to titre by a *S. thompson*, phase 2 (1,5) serum and single factor 5 serum.

Therefore the antigenic formula of this type was 11 : b : 1,5. As a *Salmonella* type with this formula is as yet not described, we propose the name *Salmonella leeuwarden*.

We are indebted to Dr. F. KAUFFMANN, International Salmonella Centre (Copenhagen) for the confirmation of our findings.

Summary.

A new *Salmonella* type *Salmonella leeuwarden*, isolated from the stool of a baby, is described.

The antigenic formula is 11 : b : 1,5.

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THE EFFECTS OF 2,4-DINITROPHENOL ON THE OXIDATION OF FATTY ACIDS BY *PSEUDOMONAS AERUGINOSA* [1])

by

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(Received October 15, 1959).

At the present time very little is known concerning the oxidation of fatty acids by pseudomonads and nothing has been reported concerning the effects of phenols on this process. JAZESKI *et al.* (1950) believe that two different enzyme systems are involved in the oxidation of compounds of the saturated fatty acid series. One system is specific for the short chain and one for the long chain fatty acids.

The objective of the present investigation was to determine the effects of 2,4-dinitrophenol (DNP) on the oxidation of fatty acids by *Pseudomonas aeruginosa.*

EXPERIMENTAL PROCEDURE.

A pure culture of *Ps. aeruginosa* isolated from a spoiled emulsion oil and identified by its morphological and biochemical properties was used throughout this research. The culture was transferred and checked for purity at regular intervals. Stock cultures were maintained under refrigeration on trypticase soy agar (Difco) [2]) slants.

The cells used in the oxidative studies were grown on a medium of the following composition: bacto-peptone (Difco) [2]), 20 g; glucose, 10 g; Na_2SO_4, 1.5 g; $MgSO_4$, 1.5 g; distilled water, 1 l; pH adjusted to 7.2–7.4. Cells were grown statically in 300 ml of this broth for 24 hr at 37°C., harvested by centrifugation at 3500 rpm, washed in

[1]) This investigation was supported by a Fellowship from the Anderson Oil and Chemical Company, Inc., Portland, Connecticut.

[2]) Difco Laboratories, Inc., Detroit, Michigan.

M/50 NaOH-KH_2PO_4 buffer, pH 7.0, and resuspended in 100 ml of fresh buffer. The suspension was then shaken on a laboratory shaker at 25°C. for 10–12 hr in order to lower the high rate of endogenous respiration initially encountered in oxidative studies using freshly harvested cells. After this period the cells were centrifuged at 3500 rpm, resuspended in sterile distilled water and adjusted to an optical density of 1.0 using a Klett-Summerson photoelectric colorimeter at 400–460 mμ (blue filter).

The fatty acid substrates used in these studies were of the highest possible purity (99.5 per cent, Eastman) [1]). For the oxidative experiments the acids were prepared as 0.05 percent stock solutions with M/5 NaOH-KH_2PO_4 buffer, pH 7.0. Some of the longer chain fatty acids formed colloidal suspensions and were triturated in a hand homogenizer in order to obtain evenly dispersed solutions. The stock solutions of the inhibitor were prepared in the desired concentrations with M/5 NaOH-KH_2PO_4 buffer, pH 7.0. All stock solutions were stored under refrigeration.

The cell suspensions were used in oxidative studies following standard Warburg manometric techniques (Umbreit *et al.*, 1957). The reaction flasks contained 0.5 ml of the fatty acid soap in one side arm and when used, 1.0 ml of the inhibitor in the other side arm. The center well contained 0.1 ml of 20 per cent KOH absorbed onto a small square of fluted filter paper. One ml of cell suspension plus sufficient M/50 NaOH-KH_2PO_4 buffer, pH 7.0, to give a total flask volume of 3.1 ml was added to the main well.

The substrates and inhibitor were tipped in simultaneously from the sidearms at zero time. Oxygen uptake was then followed for at least 6 hr in an air atmosphere. Autorespiratory values were determined in the presence and absence of DNP but were not incorporated into the oxidative data. Oxygen uptake was calculated and recorded as μl of oxygen per mg dry weight of cells. Each experiment was repeated several times in order to verify the results.

The effect of DNP on the growth of *Ps. aeruginosa* in a fatty acid-inorganic salts medium was also studied. A buffered salts solution was prepared which consisted of KH_2PO_4, 0.2 g; $MgSO_4$, 0.2 g; $(NH_4)_2SO_4$, 1.0 g; Na_2SO_4, 0.1 g; $Fe(NH_4)_2SO_4$, 0.1 g; M/5 NaOH-KH_2PO_4 buffer, pH 7.0, 1 l. After the salts were dissolved, the solution was filtered through Whatman #2 filter paper in order to

[1]) Eastman Kodak Co., Rochester, New York.

remove the insoluble precipitates which were formed. The fatty acids were prepared to a 0.1 per cent solution and dispensed as 100 ml aliquots into four 250 ml Erlenmeyer flasks. Two sets of control flasks were also prepared. One contained the buffered inorganic salts solution with no fatty acid, and the other the fatty acid salt prepared in M/5 NaOH-KH_2PO_4 buffer, pH 7.0. Two flasks of each of these sets were then supplemented with enough of the inhibitor to give that concentration which caused optimum stimulation of oxygen uptake in the Warburg studies. These flasks were then stoppered with cotton plugs and sterilized in the autoclave at 15 pounds pressure and 121°C. for 15 min.

The cell inocula for these flasks were prepared in the same manner as the cells for the Warburg studies, including the shaking period. The cell suspension was adjusted to an optical density of 0.1 using a Klett-Summerson photoelectric colorimeter at 400–460 mμ (blue filter). The flasks were inoculated with 0.1 ml of this suspension and then incubated on a rotary shaker at 30°C. and at 180 rpm. Duplicate standard plate counts were made at zero time and at 24 hr intervals using sterile distilled water as the diluent and plate count agar (Difco) as the medium. The plates were incubated at 37°C. for 48 hr and the colonies counted. The counts are reported as numbers of organisms per ml of culture medium. Inoculated and uninoculated controls accompanied each set of experiments and, when pertinent, are listed in the experimental data.

Results.

Table 1 records the effects of varying concentrations of DNP on the endogenous respiration of *Ps. aeruginosa*. DNP stimulates autorespiration at all concentrations with the greatest increase occurring in a concentration of 50 ppm. With the exception of 50 ppm this stimulation is preceded by an initial period of inhibition.

The effects of DNP on the oxidation of butyric acid are listed in table 2. It may be seen that 25 and 50 ppm of DNP cause a slight stimulation, which is greatest after 6 hr, while higher concentrations create a significant inhibition of oxygen consumption.

All of the concentrations of DNP produce an immediate stimulation with the caproic and caprylic acids (tables 3 and 4). The optimum oxygen uptake occurs in the range of 100 to 150 ppm of DNP and decreases as the oxidation progresses.

TABLE 1.

Effects of 2,4-dinitrophenol (DNP) on the endogenous respiration of *Pseudomonas aeruginosa.*

Concentration of DNP	μl O_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
None	6	8	15	27	33	42	—	—	—	—	—	—
25 ppm	6	9	18	30	37	47	0	13	20	11	11	12
50 ppm	7	12	21	34	40	50	17	50	40	26	21	19
100 ppm	6	9	18	31	38	48	0	13	20	15	15	14
150 ppm	5	8	17	30	37	47	–17	0	13	11	11	12
200 ppm	4	8	16	29	37	47	–34	0	7	7	11	12
250 ppm	4	8	17	31	38	48	–34	0	13	15	15	14

*Average values of two determinations.

Air atmosphere; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 2.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of butyric acid by *Pseudomonas aeruginosa.*

Concentration of DNP	μl O_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	6	10	21	30	37	52	—	—	—	—	—	—
None	14	43	98	160	186	220	—	—	—	—	—	—
25 ppm	15	44	99	161	189	239	7	2	1	1	2	9
50 ppm	14	41	96	162	191	240	0	– 5	– 2	1	3	9
100 ppm	8	35	86	146	176	214	–43	–19	– 8	– 9	– 5	– 3
150 ppm	11	34	84	139	176	209	–21	–21	–14	–13	– 5	– 5
200 ppm	12	32	68	117	164	187	–14	–26	–31	–37	–12	–15
250 ppm	11	29	62	110	153	181	–21	–33	–37	–31	–18	–18

*Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; butyric acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 3.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of caproic acid by *Pseudomonas aeruginosa.*

Concentration of DNP	μl o_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	6	8	15	24	30	42	—	—	—	—	—	—
None	11	33	76	130	178	233	—	—	—	—	—	—
25 ppm	17	40	81	135	187	253	54	21	6	4	5	9
50 ppm	17	43	85	137	189	256	54	30	12	5	6	10
100 ppm	22	50	91	147	200	266	100	52	20	13	12	14
150 ppm	18	44	94	148	205	268	64	33	24	14	15	15
200 ppm	18	39	87	140	194	265	64	18	14	8	9	14
250 ppm	14	37	85	139	190	263	27	11	12	7	6	13

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; caproic acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 4.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of caprylic acid by *Pseudomonas aeruginosa.*

Concentration of DNP	μl o_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	3	6	12	17	21	30	—	—	—	—	—	—
None	21	55	116	162	181	237	—	—	—	—	—	—
25 ppm	25	62	127	179	188	245	19	13	8	11	4	3
50 ppm	27	62	127	173	184	239	29	13	8	7	2	1
100 ppm	34	71	135	183	195	257	62	29	16	12	8	8
150 ppm	32	67	130	179	188	249	52	22	12	11	4	5
200 ppm	32	70	132	182	187	247	52	27	12	12	3	4
250 ppm	26	67	138	183	187	250	19	22	19	12	3	6

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; caprylic acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer pH 7.0.

The oxygen uptake with capric, lauric, tridecanoic and myristic acids is increased after 6 hr by all the concentrations of DNP with the greatest enhancement occurring in the range of 100–150 ppm of DNP (tables 5–8). However in most cases this stimulation is first preceded by a period of slight inhibition.

The effects of DNP on the oxidation of palmitic acid by *Ps. aeruginosa* presents a different pattern (table 9). The concentration of 25 ppm of DNP causes a slight stimulation of oxygen uptake while higher concentrations inhibit oxidation.

The effect of DNP (100 ppm) on the growth of *Ps. aeruginosa* in a medium containing a fatty acid as the sole source of carbon is recorded in table 10. The growth of *Ps. aeruginosa* in capric acid-salts medium is inhibited by the same concentration that causes stimulation of oxidation.

Discussion.

The data presented herein contain some basic information concerning the oxidation of fatty acids by *Ps. aeruginosa*. The absence of an initial lag in the uptake of oxygen (tables 2–9) indicates that *Ps. aeruginosa* probably has a constitutive enzyme system for the oxidation of saturated aliphatic fatty acids. This property may be common to the genus *Pseudomonas* since it has been shown that 11 species of pseudomonads oxidize capric and pelargonic acids without an initial lag (Silliker and Rittenberg, 1951) and cell free extracts of *Ps. fluorescens* oxidize capric acid (Ivler *et al.*, 1955).

Examination of the tables reveals that the rates of oxidation of the various fatty acids by *Ps. aeruginosa* depend on the chain length of the acids. Oxidation is most rapid with lauric acid (C_{12}), while the oxygen consumption with the other acids progressively decreases from the level of the C_{12} acid as their chain lengths become shorter or longer.

Jazeski *et al.* (1950) have reported that pseudomonads possess two different enzyme systems for fatty acid oxidation; one system specific for the short chain and one for the long chain fatty acids. The effect of varying concentrations of DNP on fatty acid oxidation indicates that this assumption may not be true in every case. The oxygen uptake with the C_4 and C_{16} acids is increased slightly by 25 and 50 ppm of DNP, while with the C_6, C_8, C_{10}, C_{12}, C_{13} and C_{14} acids there is a stimulation with all concentrations of DNP.

TABLE 5.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of capric acid by *Pseudomonas aeruginosa.*

Concentration of DNP	μl o_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	7	11	23	32	40	54	—	—	—	—	—	—
None	35	86	174	188	205	242	—	—	—	—	—	—
25 ppm	28	74	162	182	200	244	–20	–14	–7	–3	–2	1
50 ppm	37	90	179	198	218	262	6	5	3	5	6	8
100 ppm	37	93	183	201	222	269	6	8	5	7	8	11
150 ppm	34	90	175	194	215	269	– 3	5	1	3	5	11
200 ppm	21	72	162	183	203	261	–40	–16	–7	–3	–1	8
250 ppm	29	81	117	195	218	272	–17	– 6	2	4	6	12

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; capric acid, 0.017 per cent flask concentration; temperature; 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 6.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of lauric acid by *Pseudomonas aeruginosa.*

Concentration of DNP	μl o_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	4	11	20	27	33	45	—	—	—	—	—	—
None	33	104	208	238	251	275	—	—	—	—	—	—
25 ppm	27	102	203	234	249	274	–18	–2	–2	–2	–1	0
50 ppm	32	108	217	238	255	286	– 3	4	4	0	2	4
100 ppm	29	108	215	235	253	286	–12	4	3	–1	1	4
150 ppm	33	105	210	234	261	294	0	1	1	–2	4	7
200 ppm	33	106	211	242	257	289	0	2	2	2	2	5
250 ppm	22	100	201	235	253	283	–33	–4	–3	–1	1	3

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; lauric acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 7.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of tridecanoic acid by *Pseudomonas aeruginosa*.

Concentration of DNP	μl o_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	1	5	12	20	29	37	—	—	—	—	—	—
None	31	78	184	206	226	263	—	—	—	—	—	—
25 ppm	31	81	189	220	248	301	0	4	3	7	10	15
50 ppm	32	83	191	223	253	307	3	6	4	8	12	17
100 ppm	40	89	203	237	266	319	29	14	10	15	18	21
150 ppm	33	83	196	231	260	314	6	6	6	12	15	19
200 ppm	28	73	191	223	252	306	–10	–6	4	8	12	16
250 ppm	28	72	190	220	249	303	–10	–8	3	7	10	15

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; tridecanoic acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 8.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of myristic acid by *Pseudomonas aeruginosa*.

Concentration of DNP	μl o_2 uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	½	1	2	3	4	6	½	1	2	3	4	6
Endogenous	5	10	18	27	34	46	—	—	—	—	—	—
None	31	77	162	198	229	256	—	—	—	—	—	—
25 ppm	27	74	158	196	228	257	–13	–4	–3	–1	0	0
50 ppm	30	76	169	204	238	267	– 3	–1	4	3	4	4
100 ppm	30	77	170	205	240	269	– 3	0	5	4	5	5
150 ppm	31	77	169	204	239	272	0	0	4	3	4	6
200 ppm	30	75	164	198	232	265	– 3	–3	1	0	1	3
250 ppm	29	74	163	198	230	261	– 6	–4	1	0	0	2

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; myristic acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 9.

Effects of 2,4-dinitrophenol (DNP) on the oxidation of palmitic acid by *Pseudomonas aeruginosa*.

Concentration of DNP	$\mu l\ O_2$ uptake per mg dry weight of cells in hours*						Per cent stimulation or inhibition in hours					
	$\frac{1}{2}$	1	2	3	4	6	$\frac{1}{2}$	1	2	3	4	6
Endogenous	3	8	15	21	28	47	—	—	—	—	—	—
None	12	25	65	103	141	218	—	—	—	—	—	—
25 ppm	13	26	66	106	147	227	8	4	2	3	4	4
50 ppm	10	23	61	100	139	222	− 8	− 8	− 6	− 3	− 1	2
100 ppm	10	22	59	96	133	214	− 8	−12	− 9	− 7	− 6	− 2
150 ppm	10	19	48	81	116	193	− 8	−24	−26	−21	−18	−12
200 ppm	8	16	30	60	89	158	−33	−36	−54	−42	−37	−28
250 ppm	7	15	29	57	81	138	−42	−40	−55	−45	−43	−37

* Average values of two determinations; values are not corrected for endogenous respiration.

Air atmosphere; palmitic acid, 0.017 per cent flask concentration; temperature, 30°C.; M/50 NaOH-KH_2PO_4 buffer, pH 7.0.

TABLE 10.

Effects of 2,4-dinitrophenol (DNP) on the growth of *Pseudomonas aeruginosa* in capric acid-salts medium.

Days	Viable organisms per ml ($\times\ 10^6$)*					
	Control 1**		Control 2***		Capric acid	
	No DNP	DNP	No DNP	DNP	No DNP	DNP
0	0.07	0.19	0.16	0.10	0.15	0.12
1	15	8	0.80	0.82	2420	1840
2	19	8	0.59	0.61	3180	1580
3	24	7	0.60	0.52	4500	1790
4	28	5	0.82	0.45	3850	1030
5	32	8	0.72	0.89	4750	770
6	31	11	0.60	0.96	2810	425
7	32	9	0.72	1.02	2100	322

* Average values of two determinations made on each duplicate flask by standard plate count technique.
** Buffered inorganic salts solution with no capric acid.
*** Buffered capric acid solution with no inorganic salts.

Capric acid, 0.1 per cent; DNP, 100 ppm flask concentration; M/5 NaOH-KH_2PO_4 buffer, pH 7.0; temperature, 30°C.; agitated at 180 rpm.

It may be noted that even though DNP stimulates oxygen uptake the compound has an inhibitory effect upon multiplication of the organisms (table 10).

Summary.

The effects of varying concentrations of 2,4-dinitrophenol on the oxidation of a series of saturated, aliphatic fatty acids by *Ps. aeruginosa* were studied. The oxidation of C_4, C_6, C_8, C_{10}, C_{12}, C_{13}, C_{14} and C_{16} acids is stimulated by certain concentrations of this inhibitor. However, the concentration of 2,4-dinitrophenol which causes stimulation of oxygen uptake with capric acid does not produce an increase in numbers of the organisms in a medium containing the fatty acid as the sole carbon source.

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MURINE TUBERCULOSIS IN A CAT

by

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(Received December 3, 1959).

Introduction.

Natural infection with murine tuberculosis was found by Wells (1937) in voles (*Microtus agrestis*), whilst the same investigator (1946) encountered this bacterium in the bank-vole (*Clethrionomys glareolus*), in the woodmouse (*Apodemus sylvaticus*) and as far as concerns animals other than rodents only in the shrew (*Sorex araneus*). By Wlodzimierz Nicewicz (1952) murine tuberculosis was detected in the continental vole (*Microtus arvalis*), a species which is closely related to the common vole.

The official name of the bacterium, which caused murine tuberculosis, was established as *Mycobacterium tuberculosis* var. *muris* Brooke (Wells, 1953). Natural infections in animals other than the above mentioned are not published, whereas in the Netherlands cases of murine tuberculosis are unknown.

Case history.

In December 1956 a cat (killed by strychnine-injection) was received at the State Serum Institute (now Central Veterinary Institute, Department Rotterdam), which showed lungs with tuberculosis-like lesions and a similar focus in the mesenterial lymph nodes.

Microscopic investigation of the lesions showed the presence of acid-fast rods on which the later observed peculiar form was not yet detected. After the usual pretreatment, this material was inoculated on Löwenstein media and on Stonebrink media, both with and without glycerol. No growth was however obtained.

Another part of the material from the cat was injected into two

albino guinea pigs (1 and 2). These animals were skin tested with tuberculin for the first time $3\frac{1}{2}$ weeks after infection, in a way previously described by us (1959). The animals were slightly allergic to bovine tuberculin, and one of the two showed a very small reaction to avian tuberculin. The animals were in perfect health. At the second skin test — performed nine weeks after infection — the reactions were larger than the first time. Both animals were sacrificed.

Post mortems were negative, except for a very slight enlargement of the regional lymph nodes. Cultures were made from spleen and regional lymph nodes on the above mentioned media.

This time, growth was first detected after about $2\frac{1}{2}$ months incubation on Stonebrink medium without glycerol and 2 months later, a few colonies were also visible on the Löwenstein medium without glycerol. Both types of media with glycerol showed no growth at all.

This lack of pathogenicity for guinea pigs which became allergic to mammalian tuberculin, combined with the very slow growth, made us suspect that, possibly, in this case we were dealing with murine tuberculosis.

Microscopic examination strengthened this supposition, because we saw the peculiar forms as described by GRIFFITH (1942), to wit curved forms abounding, among them shepherd's crooks, bacteria in the form of a sickle, spiral forms and S-types.

Cultural characteristics. The colony type we got in the Stonebrink medium was different from that generally obtained from bovine, human or avian tubercle bacteria. The colonies were pearly white, smooth to slightly granular, somewhat lower than hemispherical and abruptly rising from the surface of the medium. Avian tubercle bacteria form glistening hemispherical colonies on this medium, mostly buff coloured, bovine tubercle bacteria form colonies with a wide frilled margin and a rather low central bud, whilst human tubercle bacteria generally develop into a bread crumb-like colony with a frilled margin, mostly comparatively narrower than that seen in bovine colonies.

According to TOPLEY-WILSON (1955) the murine bacterium grows in plain trypsinized broth. We obtained the same result and got very small pellicles on the surface of this medium. We also tried the possibility of growth on potato broth without glycerol. On this medium growth is rather good: on the potato, granular colonies are formed as a pellicle, starting from the potato, spreading along the

surface of the fluid part of this medium. These pellicles are more extensive than those on the plain trypsinized broth.

P a t h o g e n i c i t y f o r r a b b i t s. A rabbit was inoculated intravenously with 0.01 mg suspension of the bacterium. Post mortem investigation of this animal, performed 4 months later showed no lesions at all, a fact corresponding well with data given by GRIFFITH (1942).

The whole of the results — obtained by our microscopic and cultural examinations, together with those got in the animal experiments — led us to the conclusion, that the isolated strain could be diagnosed as *Myc. tuberculosis* var. *muris*. At this stage of our investigation we asked the veterinary surgeon, who sent the cat, if this animal used to eat voles and received an affirmative answer.

F u r t h e r a n i m a l e x p e r i m e n t s. GRIFFITH and DALLING (1940) infected calves and found the murine type of tuberculosis suitable for vaccination purposes. Moreover, murine tuberculosis is sometimes mentioned, as one of the possible causes for the development of the so called non-specific reactions on mammalian tuberculin in cattle. So, for this reason, we infected a calf with murine tuberculosis, by injecting it in an ear vein with a suspension in saline of a culture on Stonebrink egg medium. The single comparative tuberculin-test was performed on the animal twice, to wit 4½ months and 6½ months after infection. This comparative test is done in the Netherlands by using 0.1 ml 0.4 mg protein/ml strength avian PPD tuberculin and 0.1 ml 1.5 mg protein/ml strength bovine PPD tuberculin. The increase in thickness of the skinfold as measured by calipers was the first time for avian tuberculin 4.3 mm and for bovine tuberculin 7.6 mm. The second time these reactions were 4.7 mm and 5.5 mm resp. This allergy is rather more distinct for the avian tuberculin than would be expected for an animal inoculated with a mammalian strain and if an animal with suchlike reactions should be encountered in serial examination it would have been regarded as a non-specific reactor. Post mortem investigation of the calf 7 months after infection did not show gross lesions at all, but cultural examination of the bronchial and mediastinal lymph nodes on Stonebrink media without glycerol was positive after a few months and showed the already described type of colonies. Three guinea pigs (5, 6 and 7) were also inoculated with a suspension of the calf lymph nodes. These guinea pigs, together with two previous-

ly infected animals (3 and 4) — inoculated with about 0.2 mg (wet weight) bacteria obtained from cultures of organs from the two first infected guinea pigs (1 and 2) — were submitted to comparative tuberculin skintesting with suitable doses of tuberculin (80 TU avian, 75 TU bovine tuberculin). The results were as follows:

Results of tuberculination.

Guinea pig	Date of infection	9–12–57		9–4 –58		14–7–58		7–11–58		12–3–59		diameter in mm	
		av.	bov.	av.	bov.	av.	bov.	av.	bov.	av.	bov.	av.	bov.
3	19–10–57	+	++++	++	+++	++	+++			+	+++	16	19
4	19–10–57	|	+++	—	++	.	++			|	+ |	8	15
5	24– 7–58							.	++ |	+	+++ |	10	20
6	24– 7–58							+	+++	+	++++	13	22
7	24– 7–58							.	++ |	+	++++	12	20

The reactions to avian tuberculin in these animals tend to be somewhat larger than those generally seen in guinea pigs inoculated with a human or a bovine tuberculosis strain.

No gross lesions were detected at post mortem examination of these five animals in connection with the infection, but guinea pig No. 3, the animal with the more non-specific type of reactions, showed signs of chronic nephritis.

Microscopic investigation of smears, made from the regional lymph nodes and the spleen, showed the presence of a few acid-fast rods, mostly rather short and only in the smears from guinea pig No. 3 were a few curved observed. Cultural examination of the same material only led to the development of colonies in the case of guinea pig No. 3, 5, and 6. So it was proved again that the pathogenicity for guinea pigs is very small.

In the literature the pathogenicity of murine tubercle bacteria is only mentioned for several species of rodents and for rabbits, shrew and calf (Wells, 1946). Besides very extensive vaccination trials have been performed on human beings (Committee Report Brit. Med. J. No. 4964, February 25, 1956).

Pathogenicity for carnivores, however, seems unknown. Therefore it was very much appreciated, that we got the opportunity to use some ferrets (*Putorius putorius furo*). We tried to get an impression of the pathogenicity of the murine strain for this animal, because for the closely related pole cat (*Putorius putorius*) voles form a part of the natural diet.

For this purpose each ferret was fed with 25 mice, which mice

after killing had been injected with 1 ml of a suspension of murine tubercle bacteria, obtained from a Stonebrink egg medium slope.

This feedings of the ferrets with mice took place in a period of seven weeks. The first ferret was autopsied two months after the last mouse was consumed and almost four months after the beginning of the feeding with infected mice. The other four animals were sacrificed two months later.

All animals were in excellent condition and only one of them had a tuberculous focus with a caseous centre in a mesenteric lymph node.

Positive cultures, however, were obtained either from spleen, lungs and/or mesenteric lymph nodes from each of the ferrets.

Discussion.

Our findings confirm the very slight pathogenicity of the murine tubercle bacteria for guinea pig, rabbit, and calf. Moreover the pathogenicity for the ferret is very low, consequently wild living related species (weasel, pole cat, stoat), whose natural diet consists partly of voles, probably will not become ill when they consume voles with murine tuberculosis, and the natural equilibrium between those predatory animals and their prey will not be destroyed. On the other hand the finding of a case of a naturally infected cat demonstrates the possibility of greater pathogenicity for other species than those already mentioned in the literature.

Attention is drawn to the fact that the primary isolation of the vole-bacterium succeeded only due to the elaborate method of comparative tuberculin skin-testing of the inoculated albino guinea pigs and the use of Stonebrink medium.

On the Löwenstein medium without glycerol, first growth was observed 4½ months after inoculation, when usually the media will have been discarded already, as negative.

It seems quite possible, that in fact vole infection is more common than it is supposed to be, but not detected because investigators omitted skin-testing of inoculated guinea pigs and used too poor media.

Summary.

A case of tuberculosis in a cat, caused by the vole-type tubercle bacterium is described. Pathogenicity tests with the obtained strains

were performed on guinea-pigs, a rabbit, a calf and ferrets. In contrast with the findings in the cat the vole-bacterium proved to be only slightly pathogenic for these animals. The possible significance of the observed facts is discussed.

Acknowledgement.

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ISOLATION AND ANTIGEN PRODUCTION OF HERPES SIMPLEX VIRUS IN THE CHORIOALLANTOIC MEMBRANE OF DUCK EMBRYOS

by

R. GISPEN and B. SAATHOF

(Received February 22, 1960).

Duck embryos as well as chick embryos can be used for the propagation of viruses and rickettsiae. There is no record of systematic comparison of both species in this regard.

For a number of years we have utilized duck eggs as well as chicken eggs for growing various rickettsiae and the viruses of smallpox, vaccinia, cowpox, ectromelia, herpes simplex, psittacosis, lymphogranuloma venereum, mumps and influenza. With few exceptions eggs of both species gave much the same results. Chorioallantoic membranes (CAM) of chick embryos are slightly more susceptible to vaccinia virus, whereas differences between focal reactions of various pox viruses on the CAM are more obvious in chicken eggs than in duck eggs.

For herpes simplex virus, however, duck embryos are sometimes to be preferred to chick embryos. The advantages concern isolation of natural virus and production of CF antigen.

Results obtained with eggs of both species in laboratory diagnosis are compared here. In a later stage suckling mice were used for virus isolation, as recommended by Kilbourne and Horsfall (1951), Cath (1955) and Lennette and Van Allen (1957). Tissue cultures were used in a few cases.

Methods.

Virus isolation. Samples are sent to the laboratory in sterile capillaries, slides or swabs. The material is emulsified in

1—2 ml of buffered broth, pH 7.2, with 400 U penicillin and 100μg streptomycin per ml. The suspension is inoculated on the CAM of 11 days old duck and chick embryos (0.1 ml), intracerebrally into one-day old suckling mice (0.03 ml) and on tissue cultures of the T_1 cell line[1]) and monkey kidney cells[2]).

The inoculated eggs are incubated at 35° C. Reactions on CAM are read 2, 3 and 5 days after inoculation through the rectangular hole in the shell by means of a mirror lamp attached to the forehead. Membranes with typical focal reactions are examined histologically for intranuclear inclusions in epithelial cells of the foci. No blind passages were carried out.

Suckling mice are observed twice a day for 2 weeks after intracerebral inoculation. Diseased animals are sacrificed and examined histologically for meningomyelitis, whereas an emulsion of the brains is inoculated on CAM of duck embryos to confirm the herpes simplex diagnosis.

Tissue cultures with cytopathogenic degeneration are examined histologically for intranuclear inclusions and subinoculated into eggs.

Virus titration. Ten-fold dilutions of the virus are inoculated on the CAM of 2-4 eggs. Focal reactions are read after 3 days. A CAM with more than 3 typical foci is considered as positive. The titer corresponds with highest dilution being positive in half or more of the eggs.

CF antigen. CAM of eleven days old duck embryos are inoculated with 0.1 ml of a 1 per cent suspension of infected membranes. The eggs are incubated at a temperature of 35° C. during a period of 5 days. Embryos being dead within 2 days after the inoculation are discarded. The remaining embryos are put into a refrigerator at 4° C. during a few hours at the end of the incubation period.

Membranes are selected for well developed reactions. Each of the selected membranes is examined separately for absence of bacteria and stored in a deepfreezer at – 25° C.

The membranes are pooled and emulsified to a 20 per cent suspension in buffered saline pH 7.2 by means of a Waring Blender. The homogenized suspension is frozen at – 70° C. and thawed. This cycle is repeated 4 times. The suspension is centrifuged at 2500 rpm

[1]) Established by VAN DER VEEN *et al.* (1958) from human kidney.

[2]) Tissue culture inoculations were carried out in this laboratory by Dr. J. G. KAPSENBERG.

(1600 × g). The supernatant is used as CF antigen after addition of 0.01 per cent merthiolate. Normal antigen is prepared from non-infected membranes in the same way. Antigens are prepared from infected and non-infected CAM of chick embryos for comparison.

A n t i s e r a. Guinea-pigs are infected intracerebrally with 0.15 ml of a concentrated virus suspension. Diseased animals are sacrificed. The brains are homogenized to a 10 per cent suspension in saline. A ten-fold dilution of the suspension should give confluent lesions on the CAM of embryonated eggs. Guinea-pigs are injected intraperitoneally with 1 ml of the 10 per cent brain suspension. The injection is repeated 7 times with intervals of 5 days. The animals are bled 10 days after the last injection. The serum is stored without preservative at – 25° C.

C o m p l e m e n t f i x a t i o n. CF tests are carried out in plastic plates. The optimal antigen dose is determined in a block-titration with two-fold dilutions of antigen and antiserum. The antigen titer corresponds to the highest antigen dilution giving complete fixation in 1 : 4 serum dilution. The antigen is used in a concentration corresponding to at least 4 × the concentration of the antigen titer dilution. 2 M.H.D. of complement are used in the test. The hemolytic system consists of 2 M.H.D. amboceptor and 2 per cent sheep erythrocytes mixed in equal volumes. Fixation is carried out at 4–6° C. overnight.

H i s t o l o g i c a l e x a m i n a t i o n. Bouin's fluid is used as fixative. Hematoxylineosin stain is used [1]).

Results.

V i r u s i s o l a t i o n. It was attempted to isolate virus from vesicle fluid or scrapings from lesions on skin and mucous membranes of suspected cases. Various methods were used: inoculation on CAM of duck and chick embryos, on monolayers of tissue cultures and intracerebral injection into suckling mice.

Twenty two cases showing positive results with at least one of the methods used are mentioned in table 1. Duck CAM and suckling mice gave considerably more positive results than chick CAM or tissue cultures. Each of both former methods showed a sensitivity twice as high as that of the two other methods.

The superiority of duck CAM to chick CAM is shown also in table 2.

[1]) Histological techniques were carried out in this institute by Dr. H. H. Vink.

TABLE 1.

Comparison of various methods for herpes simplex virus isolation.

No.	Simultaneous inoculation of the sample in				
	Chorioallantoic membrane		Suckling mice intracerebrally	Tissue culture	
	duck	chicken		cell line T_1	monkey kidney cells
740	+	+			
1089	+	+			
1130	+	+			
1376	+	+			
1818	+	+			
1887	+	+			
1927	+	—			
57– 10	+	+			
57– 169	+	+			
57– 283	+	—	+		
57– 514	—	—	+		
57–1135	+	—			
58– 551	—	—	—	+	—
58– 865	+	—	+	+	
58–1413	+	—			
58–1576	+	—	+	—	—
58–1855	+	—	+	—	—
58–1980	—	—	+	—	—
59– 167	+	—	+	—	
59– 839	+	+	+	+	+
59–1294	+	—	+	+	—
59–1789	+	—	+	+	+
Total of positives	19	9	10	5	2
Total of samples	22	22	11	9	7

+ = typical reactions in first passage.

In 10 out of 22 cases the duck CAM was positive, whereas the chick CAM remained negative. The reverse did not occur.

The sensitivity of intracerebral inoculation into suckling mice was not different from that of duck CAM. Nevertheless suckling mice were positive in two cases where duck CAM was negative (table 3). The reverse did not happen. Suckling mice might be slightly more sensitive than duck eggs.

Herpes simplex virus isolated and propagated on duck membranes

TABLE 2.

Results of virus isolation on duck and chick chorioallantoic membranes in the cases of table 1.

Chorioallantoic membrane		Number of cases
duck	chicken	
+	+	9
+	—	10
—	+	0
—	—	3
Total		22

TABLE 3.

Results of virus isolation in duck CAM and cerebrum of suckling mice in the cases of table 1.

Duck CAM	Suckling mice	Number of cases
+	+	8
+	—	0
—	+	2
—	—	1
Total		11

TABLE 4.

Titration of herpes simplex virus of various passage numbers in duck and chick eggs.

Strain no.	Egg passage of virus suspension	Log. virus titer on CAM		Egg passage of virus suspension	Log. virus titer on CAM	
		duck	chicken		chicken	duck
58–865	D_1	3		C_1	< 1	
57– 10	D_1	3		C_1	1	
1887	D_1	2		C_1	1	
1818	D_1	2		C_1	< 1	
1089	D_2	5	4	C_2	2	2
1376				C_3	3	3
740	D_3	4	4			
1376	D_4	4	4			
740				C_5	2	2
740				C_6	2	3
740				C_8	3	3

D_1, D_2 etc. indicate the number of passages in duck CAM.
C_1, C_2 etc. indicate the number of passages in chick CAM.

can be more rapidly adapted and shows higher virus titers than virus isolated and propagated on chick membranes (table 4).

The virus produces relatively large focal reactions even in the first or second passage on duck CAM, whereas this takes several more passages in chick embryos. It is noteworthy that titrating egg-adapted virus on duck or chick embryos makes no difference. The sensitivity of eggs of both species for egg-adapted virus is equal, though virus production per focal reaction in chicken eggs is apparently lower than in duck eggs.

Reactions in duck CAM. Focal reactions of 58 strains of herpes simplex on duck CAM were examined. These are larger in the first and second passage on duck CAM than in chick membranes. Epithelial proliferation is more extensive and intranuclear inclusions are more numerous than in infected chick membranes.

The inclusions were always easily found at the first and later passages of the 58 virus strains in duck CAM. Sometimes they were scanty in chick CAM, whereas the same strain produced inclusions in large quantities after inoculation on duck membranes.

Reactions on duck CAM are progressive during a period of 5 days after inoculation, maximal proliferation with glossy appearance being obtained on the 5th day. On the contrary, reactions in chick eggs tend to decrease after 3 days of incubation. This difference is

TABLE 5.

Titration of herpes simplex virus on duck and chicken chorioallantoic membrane with readings after 3 and 5 days.

Strain no.	Egg passage of virus suspension	Log virus titer on chorioallantoic membrane					
		duck			chicken		
		Reading after		Difference log titer (5-3 days)	Reading after		Difference log titer (5-3 days)
		3 days	5 days		3 days	5 days	
740	5	2	2	0	2	1	—1
740	6	3	4	+1	2	2	0
740	8	3	3	0	3	2	—1
1089	2	5	6	+1	4	4	0
1089	2	2	3	+1	2	1	—1
1376	3	3	2	—1	3	3	0
1376	4	4	5	+1	5	5	0
1887	2	2	3	+1	1	1	0

reflected in readings made after 3 and 5 days. CAM of duck eggs show frequently higher virus titers after 5 than after 3 days. The reverse holds true for chick membranes (table 5).

CF antigen. CF antigens prepared from duck and chicken eggs were tested in block-titrations with antiherpes serum. The antigen titers were considered as a measure for the antigen production in the original membranes.

The frequency distribution of titers determined for antigens from duck and chick origin is shown in table 6. Antigens made from infected duck membranes have a considerably higher titer than

TABLE 6.

Frequency distribution of various herpes simplex antigens prepared from duck and chick eggs according to their antigen titers obtained in CF tests with 1 : 4 diluted herpes simplex antiserum [1]).

Antigens prepared from CAM	Frequency distribution of antigens according to titer values — Antigen titers								Total number of antigens	Average antigen titer
	<5	5	10	20	40	80	160	320		
duck	1	3	4	10	4	8		1	31	44
chicken	8	7	2	1					18	4

[1]) Herpes simplex antiserum prepared in guinea-pigs with strain 432. Serum titer = 64.

TABLE 7.

CF titers of herpes simplex antigens prepared simultaneously with the same virus strain and technique from duck and chick eggs. Serum and serum dilution as in table 6.

Strain	Number of days between inoculation and harvest	Titers of antigens prepared from eggs: duck	chicken
1089	3	10	5 ; < 5
1089	5	20	5 ; < 5
1130	5	80	< 5
1376	5	80	10
1376	5	80	< 5
740	5	20	5
Barbier [1])	5	40	< 5
Barbier	5	80	< 5

[1]) The Barbier strain isolated by Dr. H. A. E. van Tongeren was received from Prof. J. D. Verlinde.

antigens from chick eggs. The mean titers of both are in the proportion of about 10 : 1.

A number of antigens were prepared simultaneously with the same virus strain and technique from duck and chick eggs. This resulted in the same difference of antigen titer (table 7).

The species from which the eggs are derived, selection of infected membranes and an interval of 5 days between inoculating and harvesting the membranes are important to obtain good results.

Discussion.

Corneal inoculation into rabbits as a classic method for isolating herpes simplex virus is usually replaced by inoculation on the chorioallantoic membrane of chick embryos. The intracerebral inoculation into mice (Blanc and Caminopetros, 1921) has no advantage in comparison to the egg method. Kilbourne and Horsfall (1951) demonstrated the superiority of intracerebral inoculation into suckling mice. Intracerebral inoculation was found to be a more sensitive method than the intraperitoneal route. In our laboratory, where one-day old mice are used, intracerebral infection has produced optimal results.

In this regard duck chorioallantoic membrane was almost as effective as intracerebral inoculation into one-day old mice. This is of practical importance in laboratories with limited provisions for mice breeding.

Higher virus concentration and continued antigen production after the third day of incubation are also advantages where duck eggs are concerned. The higher yield of CF antigen is probably due to the more progressive growth of virus.

Summary.

Isolation of herpes simplex virus and production of CF antigen was tried in chorioallantoic membranes of embryonated duck eggs.

Duck CAM is twice as sensitive as chick CAM to natural virus and has almost the same susceptibility as suckling mice after intracerebral inoculation.

Herpes simplex virus can be more rapidly adapted to duck eggs than chicken eggs. The first few subcultures result in higher virus titers if the virus is grown in duck CAM. No difference of virus titer

is found, however, if the same sample of egg-adapted virus is titrated in duck and chick membranes.

Herpes reactions are in the first few passages more progressive on duck CAM than on chick membranes and show continued proliferation between 3–5 days after inoculation. Herpes reactions on chick membranes tend to decrease after the 3rd day of incubation.

Intranuclear inclusions of the Lipschütz type are always found in large numbers in reactions of the first passage on duck membranes, even when they are scanty in parallel membranes of chick eggs.

CF antigens of herpes simplex virus were prepared from selected duck chorioallantoic membranes harvested 5 days after inoculation.

CF antigens of herpes simplex virus prepared from duck eggs are reproducible and show high antigen titers averaging about 10 times those of chick egg preparations.

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SALMONELLA AND *ARIZONA* INFECTIONS IN REPTILES IN THE NETHERLANDS

by

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Salmonella and *Arizona* are closely related groups of *Enterobacteriaceae*. It is, therefore, understandable that CALDWELL and REYERSON (1939) when for the first time they isolated from a Gilamonster (*Heloderma suspectum*) and a Chuckawalla (*Sauromalus ater*) a micro-organism that later appeared to be an *Arizona*, proposed the name *Salmonella dar-es-salaam*, var. *arizona*, on account of the liquefaction of gelatin, which also occurs in *S. dar-es-salaam*. Later, KAUFFMANN (1941) called this organism *S. arizona*. CALDWELL and REYERSON observed fermentation of lactose and liquefaction of gelatin, which still form the biochemical basis of the differentiation between the *Arizona* and the *Salmonella* groups.

The identification of the various serotypes in the *Arizona* group is mainly the work of EDWARDS and his co-workers (EDWARDS *et al.*, 1943, 1947, 1956a, 1959; PELUFFO *et al.*, 1942; LE MINOR *et al.*, 1958).

In America, *Arizona* bacteria have so far most frequently been isolated from turkeys, hens and eggs. However, they have repeatedly been found in patients with intestinal disorders, without other pathogenic bacteria being demonstrable (EDWARDS, 1945; MURPHY and MORRIS, 1950). In a study carried out by EDWARDS, McWORTHER and FIFE (1956b) on 87 cultures isolated from man, the high percentage of isolations from the blood of patients leads to the conclusion that *Arizona* infections in man are at least as serious as, if not more so than *Salmonella* infections. The findings of HINSHAW and McNEIL (1944, 1946) show that *Arizona* bacteria may be pathogenic for reptiles.

The *Arizona* group is characterized by the following qualities: Gram-negative, motile rods, which produce acid and gas from glucose. They produce H_2S, ferment lactose in a longer or shorter time, while gelatin is liquefied – though often slowly. Due to their definite serological relationship with *Salmonella* O and H antigens, most cultures show a positive agglutination with polyvalent *Salmonella* serum. A study on snakes, by LE MINOR, FIFE and EDWARDS (1958) revealed that a high percentage of the *Arizona* bacteria show fermentation of lactose in 24 hours, combined with gas formation; 20.8% of the strains isolated by them produce indole; 3.6% ferment saccharose, and 1% grow in the KCN medium. They used in their investigation the lysine-decarboxylase test to distinguish *Escherichia freundii* from the urea-negative, H_2S-positive, motile cultures.

PERSONAL INVESTIGATIONS.

In connection with a study on the cause of death of captive animals in the Netherlands, approximately 200 reptiles have been autopsied in the past two years. Material from the liver, heart and intestine of the vast majority of these animals was smeared on brilliant-green-phenol-red agar plates. The lactose-negative colonies and those which slowly ferment lactose were further examined. When pure cultures had been obtained these were inoculated into glucose, sucrose, lactose, dulcitol and often into sorbitol, and the motility, indole formation, H_2S production and urea fermentation were also studied. Afterwards LEIFSON's malonate medium was added. This compound is not fermented by *Salmonella*, in contrast to the positive action by *Arizona* and *Hafnia*. The two latter species can be differentiated on the ground of the lactose fermentation and the gelatin liquefaction of the *Arizona* group.

The isolated strains were sent to the Laboratory for Zoonoses and Pathological Anatomy of the National Institute of Public Health, Utrecht for determination.

Using the above-mentioned method, a number of *Arizona* types were isolated. Their characteristics are presented in Table 1.

The strain from the last-mentioned rattlesnake (*Crotalus terrificus*) was found to be a new serotype.

Besides *Arizona* bacteria, Salmonellae were isolated from several of the reptiles examined.

A striking feature was that, apart from *S. typhimurium*, various types were found that are very rarely observed in the Netherlands.

TABLE 1.

Arizona types from reptiles.

Host	Serotype
Chameleon dilepis	1, 4; 1, 2, 6
Chameleon spec.	28: 32; 28
Crotalus terrificus	1, 4; 1, 2, 6
Crotalus terrificus	26; 33: 31
Dasypeltis scabra	24: 23–31
Ahaetulla nasuta	26; 23: 32
Vipera berus	30: 23–31 (and *S. newport*)
Python reticulatus (feces)	not determined
Crotalus terrificus (feces)	1, 4; 1, 2, 6
Python reticulatus	10a, 10b: 1, 3, 11
Coluber gemanensis	23: 33–25
Crotalus atrox	1, 33: 23:–
Crotalus atrox	1, 33: 23:–
Crotalus atrox	1, 33: 23–21
Bitis gabonicus	5; 25?: 33
Crotalus terrificus	1, 4: 1, 6, 7

At the National Salmonella Centre in Utrecht, 2 of the strains were established as being new types [*S. rotterdam* and *S. blijdorp* (Guinée and Kampelmacher, 1960)].

The infection with *S. newport* in a tortoise (*Testudo graeca*) justifies a further explanation. This tortoise was sent to us by the Gemeentelijke Geneeskundige en Gezondheidsdienst (Municipal Service of Medicine and Hygiene) in Rotterdam, after the finding of an infection with *S. newport* in that city (Hemmes, 1958). The animal had long been in a family where the two young children had contracted an infection with *S. newport*. For three months, *Salmonella* could be cultured at irregular times from feces of this animal. At the end of the period, the tortoise was killed. At autopsy some redness of the mucosa of the small intestine was observed. A considerable number of *S. newport* were cultured from the coecum. Direct smear of the contents of the small intestine on brilliant-green-phenol-red agar plates produced only few colonies, whereas from the liver, bile, egg follicles, spleen and kidneys no Salmonellae could be isolated. This differs distinctly from observations in adult cattle and in man, where excretion is mainly due to the fact that the gallbladder harbours Salmonellae for a long time.

Table 2 gives a survey of the hosts and the *Salmonella* types found in them.

TABLE 2.

Host	Type
Caiman sclerops	*S. typhimurium*
Caiman crocodilus	*S. chester*
Caiman sclerops	*S. enteritidis*
Testudo graeca	*S. newport*
Tortoise (spec. indet.)	*S. saint-paul*
Chameleon jacksoni	*S. warragul*
Chameleon jacksoni	*S. blijdorp*
Chameleon jacksoni	*S. java* and *S. muenchen*
Calotes calotes	*S. typhimurium*
Calotes versicolor	*S. rotterdam*
Agame colonorum	*S. typhimurium*
Chlamydosaurus kingii	*S. typhimurium*
Basiliscus vittatus	*S. chester*
Tarentola mauritanica	*S. ngozi*
Coluber viridiflavus	*S. oranienburg*
Vipera berus	*S. newport* (and *Arizona* 30: 23–31)

Discussion.

A study on the occurrence of other *Enterobacteriaceae* than those known to be pathogenic for man appears significant in respect of public health.

The importance of such an investigation is clear in a group of animals which, originating from tropical and subtropical regions, may constitute a reservoir of this pathogenic agent.

Hemmes (1958) and Kampelmacher (personal communication) have demonstrated that reptiles are certainly capable of transmitting *Salmonella* infections to man. In a zoological garden, the conditions for spreading are much less favourable than in a family, where there is close contact with the domestic animals. In zoos, the animals are frequently kept behind glass. On account of their slight want of food, they defaecate only rarely in comparison with warm-blooded animals.

A quarantaine period with repeated faecal examination for *Salmonella* and *Arizona*, as proposed by Klein (1957) for reptiles, does not seem necessary from the viewpoint of spread of pathogenic agents in zoological gardens, provided the animals live in satisfactory hygienic conditions and in such a way that direct contact with the lookers-on is impossible.

Although much attention has been paid to this point (Boycot *et al.*, 1953; Cope *et al.*, 1955), no definite proof has as yet been found

for the possibility of infections of man through reptiles in zoological gardens. The potential risk, however, is certainly not imaginary and requires constant examination of the animals in zoos.

Summary.

A survey is given of *Arizona* and *Salmonella* types isolated from reptiles in Dutch zoological gardens.

The importance of these findings with respect to public health are discussed.

Acknowledgement.

I have to express my thanks to Dr. E. H. Kampelmacher and Mr. P. Guinée for the serological typing and the data put at my disposal.

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A NEW *SALMONELLA* TYPE (*S. NGOZI*) ISOLATED SEPARATELY IN ITS MONOPHASIC AND BIPHASIC FORM FROM A DOG AND A GECKO

by

J. MORTELMANS, R. CLAEYS and **P. A. M. GUINÉE**

(Received December 3, 1959).

During a rabies control survey of the canine population in the Ngozi province of the Ruanda-Urundi Territory (Central Africa) a culture of *Salmonella* species was isolated from the feces of a healthy native dog. The culture, isolated on SS medium after an 18 hours enrichment in tetrathionate broth showed the general morphological and biochemical characteristics of the genus *Salmonella*: Gram negative, motile rods; aerobic growth; no sporulation; rapid fermentation with gas of glucose and mannitol; lactose, sucrose, salicin and adonitol were not fermented; indol was not formed; urea not hydrolysed; H_2S was produced; the Braun KCN test was negative; nitrates were reduced to nitrites; the Voges-Proskauer test was negative; the methylred test was positive.

With the sera available in the Ruanda laboratory, the strain could not be agglutinated. Therefore the culture was submitted to Dr. VAN OYE at Leopoldville, who got an agglutination with his flagellar serum z_{10}. The culture was examined by Dr. KAUFFMANN and typed as a monophasic new *Salmonella* strain with an antigen-formula 48 : z_{10}; -.

We propose to name the new *Salmonella* type after the town Ngozi where it was isolated.

In July 1959, a culture isolated by P. ZWART (Institute for Tropical Hygiene, State University, Utrecht) from a gecko (*Taren-*

tola mauritanica [L]) was sent to the National Institute of Public Health, Utrecht, for typing. The culture was biochemically classified as *Salmonella*.

The isolated culture did not belong to O-groups A-E.

When tested in pools, O-antigen 48 was agglutinated to titre (1 : 1280). H-antigen z_{10} was agglutinated to titre (1 : 80.000) and after inhibition on Gard plates with z_{10}-serum, a second phase, which was agglutinated by H-sera 1,5 and (5) to titre (1 : 25.000 resp. 1 : 160), was found.

The antigenic formula of the new strain, which was confirmed by Dr. F. Kauffmann, is therefore 48 : z_{10}; 1.5.

Comparative examination of both cultures, isolated from the dog and the gecko, showed rapid fermentation of arabinose, dulcitol, rhamnose, trehalose, xylose, Stern's glycerol, sodium citrate and mucate. Both cultures failed to ferment inositol and i-tartrate and fermented l-tartrate within seven days.

The culture, isolated from the dog, fermented d-tartrate within seven days, whereas the culture from the gecko showed no fermentation of d-tartrate after twenty days.

Acknowledgement.

The authors wish to thank Dr. V. van Oye and Dr. F. Kauffmann for confirming their findings.

Summary.

A new *Salmonella* type, which was first isolated from a dog as a monophasic strain (48 : z_{10}; -) and some months later from a gecko as a biphasic strain (48 : z_{10}; 1.5) is described.

(Aus dem Institut für Medizin und Biologie der Deutschen Akademie der Wissenschaften, Bereich Zellphysiologie, Berlin).

DIMINUTION UND RESTITUTION DES ANOXYGENEN ENERGIEPOTENTIALS IN AEROB ZUCKERSPALTENDEN ZELLEN[1])

von

W. NORDHEIM und **F. WINDISCH**

(Eingegangen am 30. November 1959).

Am Beispiel von nicht gärenden, also ausschliesslich atmenden Hefezellen, deren RQ $\left(Q^{O_2}_{CO_2} : Q_{O_2}\right)$ in Zuckerphosphatlösung etwa 1 beträgt, konnten wir nachweisen, dass der über die Zellmembran verlaufende Phosphatwechsel obligat O_2-gebunden, d.h. mit der Zellatmung untrennbar gekoppelt ist und somit den gleichen energetischen Bedingungen unterliegt wie der Proliferationsakt, der ebenfalls nicht ohne die Mitwirkung von Sauerstoff vor sich gehen kann. — Aus der respiratorischen Gebundenheit des Phosphatwechsels vom Medium ins Plasma und seiner Hemmbarkeit durch 2,4-Dinitrophenol (9) ist kombinatorisch zu erkennen, dass es sich bei der P-Transferierung über die Zellmembran nicht einfach um eine passive Diffusion, sondern um einen Prozess aktiver Permeation handelt, der — wie LOHMANN und LANGEN (3) auf chromatographischem Wege dartun konnten — mit Phosphorylierungsvorgängen verknüpft ist. Beim Eintritt von Orthophosphat in die P-Hungerzelle findet primär eine Synthese zu höher polymeren Phosphaten statt.

Aus der Gegenüberstellung verschiedener Arten von Atmungshefen, im P-verarmten Zustand und nach 4-stündiger anaerober P-Applikation, geht mit aller Deutlichkeit hervor (Tabelle 1), dass die P-Hungerzellen, wenn sie a n a e r o b in optimale Phosphat-

[1]) Nach einem Vortrag, gehalten auf der Tagung der Gesellschaft für Physiologische Chemie vom 23.-26. September 1959 in Berlin.

TABELLE 1.

Phosphatwechsel von nicht gärenden Hefezellen.

Hefeart	P-Mangelhefe			P-Mangelhefe nach 4 stündiger Phosphataufnahme					
				a n a e r o b			a e r o b		
	mg P/ g Trs.	M-T-Reaktion[1]		mg P/ g Trs.	M-T-Reaktion[1]		mg P/ g Trs.	M-T-Reaktion[1]	
		% gefärbte Zellen	Intensitätsgrad		% gefärbte Zellen	Intensitätsgrad		% gefärbte Zellen	Intensitätsgrad
Sporobolomyces roseus	2,5	0	—	2,5	0	—	22,7	95	+ +
Torulopsis Stamm S	4,0	0	—	4,0	0	—	27,1	100	+ + +
Rhodotorula mucilaginosa	3,0	0	—	2,9	0	—	26,8	100	+ + +
Candida mycoderma	6,0	0	—	6,1	0	—	31,0	100	+ + +
Pichia membranaefaciens	2,5	0	—	2,5	0	—	21,2	90	+ +
Debaryomyces X	3,5	0	—	3,6	0	—	30,5	100	+ + +

[1]) Histochemischer Nachweis der Zellphosphate.

Intensitätsgrad: — = negativ, + = schwach positiv, ++ = mittelstark, + + + = stark.

nährlösung übergeführt werden, k e i n Phosphat von aussen in sich aufzunehmen vermögen, obwohl sie sich oxybiotisch damit übersättigen (phosphatische Überkompensation) (10).

In methodischer Hinsicht ist hier anzuführen, was für alle in dieser Veröffentlichung behandelten Untersuchungsfälle entsprechend gilt, dass die chemischen P-Analysen kolorimetrisch nach ARRHENIUS (1), die zytochromatischen Phosphatteste in Anwendung der M-T-(Methylenblau-Trypaflavin) Methode nach HAEHN und WINDISCH (6, 12) vorgenommen wurden. Das histochemische Verfahren, das auf zweifacher Säuredifferenzierung beruht, spricht spezifisch auf die Zellphosphate an, die sich als grüne bis sattgrüne Tinktionsstellen markant vom Plasma abheben. Die Bestimmung der Gärungsintensität $\left(Q_{CO_2}^{N_2}\right)$ erfolgte gravimetrisch auf makromethodischem Wege nach WINDISCH (5). Zur Überprüfung vollständiger Anoxie diente die elektrochemische O_2-Meßmethode (O_2-Galvanometrie) nach TÖDT (4).

Weit komplizierter liegen die Verhältnisse, wenn wir es mit aerob gärenden Hefen (Gärungszellen) zu tun haben, d.h. zellphysiologisch gesehen, mit jener Kategorie von Organismen, welche unter normalem O_2-Partialdruck sowohl gären als auch gleichzeitig atmen und ausserdem — im Gegensatz zu den Atmungshefen — anaerob partiell zu proliferieren vermögen. Unterzieht man derartige Gärungszellen der P-Mangelzüchtung und bringt sie danach unter Ausschluß von Sauerstoff in regenerierende Phosphatlösung, so tritt nachweislich Phosphat — trotz Einhaltung streng anaerober Versuchsbedingungen — vom Medium ins Plasma über.

Aus den Tabellen 2 und 3 gibt sich als gesetzmäßig zu erkennen, dass die verschiedenartigen Gärungszellen mit unterschiedlicher Intensität anaerob proliferieren und anaerob Phosphat in sich aufnehmen. Schwach gärende Hefeorganismen vermögen im P-Mangelzustand, wenn sie der anaeroben P-Regenerierung unterworfen werden, durchweg nur geringe Phosphatmengen ins Zellplasma zu transferieren; bei stark gärenden dagegen tritt unter gleichen anaeroben Bedingungen eine Übersättigung mit Phosphat, d.h. phosphatische Überkompensation in markanter Weise zutage. — Gärungsintensität, Potenz zur anaeroben P-Versorgung der Zelle und Anzahl der anaeroben Führungen bis zum Eintreten von Anabiose stehen in proportionaler Beziehung zueinander. Reine Atmungszellen, die anaerob kein Phosphat über die Zellmembran

TABELLE 2.

Anaerober Phosphatwechsel von schwach gärenden Hefezellen.

Hefeart	P-Mangelhefe					P-Mangelhefe nach 4 stündiger a n a e r o b e r Phosphataufnahme		
	$Q^{N_2}_{CO_2}$	Anzahl der anaeroben Passagen bis zum Eintreten der Anabiose	mg P/ g Trs.	M-T-Reaktion [1]		mg P/ g Trs.	M-T-Reaktion [1]	
				% gefärbte Zellen	Intensitätsgrad		% gefärbte Zellen	Intensitätsgrad
Sacch. exiguus	137	8	3,4	0	—	22,7	90	+ +
Torulopsis utilis, zymagen hochgezüchtet	188	11	4,5	0	—	25,6	80	+ +
Torulopsis utilis, zymagen niedergezüchtet	87	7	4,2	0	—	21,2	70	+ +
Torula cremoris	155	9	6,5	0	—	22,4	85	+ +
Torulopsis Stamm A	79	6	3,8	0	—	20,5	50	+ +
Kloeckera apiculata	66	6	3,8	0	—	17,8	60	+
Torulopsis Stamm S, zymagen umgezüchtet	46	4	4,3	0	—	15,3	60	+

[1]) Histochemischer Nachweis der Zellphosphate.
Intensitätsgrad: — = negativ, + = schwach positiv, + + = mittelstark.

TABELLE 3.

Anaerober Phosphatwechsel von stark gärenden Hefezellen.

Hefeart	P-Mangelhefe					P-Mangelhefe nach 4 stündiger a n a e r o b e r Phosphataufnahme		
	$Q^{N_2}_{CO_2}$	Anzahl der anaeroben Passagen bis zum Eintreten der Anabiose	mg P/g Trs.	M-T-Reaktion[1]) % gefärbte Zellen	M-T-Reaktion[1]) Intensitätsgrad	mg P/g Trs.	M-T-Reaktion[1]) % gefärbte Zellen	M-T-Reaktion[1]) Intensitätsgrad
Sacch. carlsbergensis Frohberg	300	19	3,5	0	—	48,7	100	+ + + +
Sacch. carlsbergensis Saaz	232	13	3,7	0	—	33,5	85	+ + +
Sacch. carlsbergensis Rasse U	273	18	3,0	0	—	43,0	100	+ + +
Sacch. cerevisiae Rasse O	275	18	4,2	0	—	43,3	100	+ + + +
Sacch. cerevisiae Rasse XII	238	13	5,2	0	—	35,1	90	+ + +
Sacch. ellipsoideus Steinberg	230	13	4,2	0	—	34,7	95	+ + +
Sacch. ellipsoideus Winninger	270	17	4,5	0	—	39,5	90	+ + +
Sacch. ellipsoideus Scharlachberg	287	17	4,3	0	—	40,2	95	+ + + +
Schizosacch. pombe	191	11	4,8	0	—	23,5	90	+ +
Sacch. pastorianus	203	11	4,1	0	—	33,5	85	+ + +

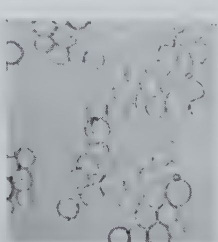
[1]) Histochemischer Nachweis der Zellphosphate.

Intensitätsgrad: — = negativ, + = schwach positiv, + + = mittelstark, + + + = stark, + + + + = sehr stark

aufnehmen, reagieren beim Fehlen von Sauerstoff sofort anabiotisch, während aerob zuckerspaltende Hefezellen — in Relation zum Grad ihres nativen Gärungsvermögens — erst über eine Reihe von anaeroben Passagen geführt werden müssen, ehe sie in den Zustand der anoxybiotischen Zytostase übergehen. Aus letzterem Umstand ist zu folgern, dass dem Typus der Gärungshefen — im Gegensatz zu den Atmungshefen — ein anoxygenes Energiepotential inhärent sein muß, welches sie befähigt, im Zustand der Anoxybiose endergonische Reaktionen zu vollführen, deren Ablauf für gewöhnlich nur in Anwesenheit von Sauerstoff vonstatten geht. (Der Begriff Energiepotential ist im vorliegenden Zusammenhang ausschließlich als biosynthetische Potenz der Zelle zu verstehen). — Wie schon angedeutet, ist die Intensität der anaeroben Proliferation und der anaeroben P-Transferierung, also die Grösse des anoxygenen Energiepotentials, vom Grad des nativen Spaltungsvermögens der Gärungszellen abhängig, welches seinerseits wiederum, vice versa, einen Maßstab für die Aktivierbarkeit des anoxygenen Energiepotentials bildet.

Die Gesetzmäßigkeit dieses Reaktionsprinzips kommt am überzeugendsten zur Geltung, wenn reine Atmungszellen zymagen umgezüchtet (2, 12) und dabei anoxygen reaktionsfähig werden, d.h. aerob zuckerspaltende Eigenschaften annehmen.

1



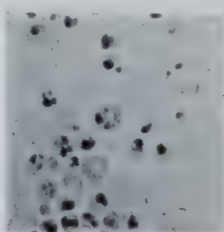


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Abb. 1. P-arm gezüchtete Atmungszellen (*Torulopsis* Stamm S) nach anaerober P-Applikation; 800 : 1.

Abb. 2. Zymagen umgezüchtete P-verarmte Atmungszellen (*Torulopsis* Stamm S) nach anaerober P-Applikation; 800 : 1.

In den vorstehenden Abbildungen kommt kontrastierend zum Ausdruck, dass P-verarmte Atmungszellen anaerob kein Phosphat aufnehmen (Abb. 1), dagegen aber ersichtlich diese Fähigkeit nach zymagener Umzüchtung erlangen (Abb. 2). Zugleich sind sie auch —

im Gegensatz zu den Atmungshefen — ohne Mitwirkung von Sauerstoff imstande, partiell zu proliferieren (7, 8).

Sämtliche Gärungshefen reagieren jedoch nur so lange anoxysynthetisch aktiv, wie sie über ein anoxygenes Energiepotential verfügen. Einem Akkumulator vergleichbar, erschöpft sich dieses im Verlauf fortgesetzter anaerober Passagen- bzw. Diminutionszüchtung, bis im Endeffekt Anabiose eintritt; umgekehrt kann die anoxygene Energieaktivität durch Sauerstoffzuführung wiederhergestellt werden.

Die energetische Ent- und Aufladung des anoxygenen Energiepotentials lässt sich in generativer Hinsicht (Proliferation) und auf histochemischem Wege (chromoanalytischer Nachweis der Zellphosphate) experimentell demonstrieren. Man geht dabei von Gärungszellen aus, die ein stark anoxysynthetisches Vermögen besitzen, und unterzieht sie der anaeroben Passagenzüchtung. Hierzu dient eine physikalisch fundierte, von uns absolut anaerob durchgebildete Verfahrensweise, die ein steriles Manipulieren unter Einhaltung normaler physiologischer Bedingungen gestattet. Die zugehörige apparative Anordnung ermöglicht es, sowohl O_2-freie Kulturen galvanometrisch kontrolliert anzulegen als auch deren Weiterimpfung nach eingetretener Zytostase ohne jeglichen Zutritt von Sauerstoff vorzunehmen. Im periodischen Prozess werden die Zellen jedesmal wieder in ein frisches anaerobes Nährsubstrat übergeimpft, so daß sie auf diese Art für längere Zeit ihre unbeeinträchtigte biologische Beschaffenheit beibehalten. Die desoxygene Versuchsgestaltung, über die wir in einer früheren Arbeit (13) bereits eingehend berichtet haben, wurde im Laufe der Entwicklung weiterhin modifiziert.

Der Gang des anoxybiotischen Züchtungsverfahrens vollzieht sich in drei aufeinanderfolgenden Etappen. Wie aus der halbschematischen Darstellung (Abb. 3) zu entnehmen ist, umfasst Vorgang I das Anlegen der O_2-freien Kultur, Vorgang II die darauffolgende Passagenzüchtung und Vorgang III den elektrochemischen O_2-Test nach Tödt. Zur Erreichung vollständig anoxygener Versuchsbedingungen benutzten wir reinsten Glühlampenstickstoff, der zur Entfernung geringster Anteile von Sauerstoff noch zusätzlich durch eine frisch bereitete $CrCl_2$-Lösung geleitet wird.

Die Versuchsgestaltung zur phasenmässigen Erfassung von Diminution und Restitution des anoxygenen Energiepotentials in Gärungszellen wurde so gewählt, dass Gärungsintensität, Vermehrung

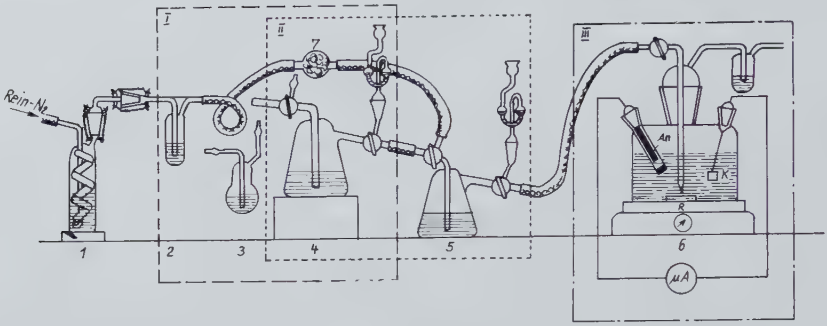


Abb. 3. Apparatur zur O_2-freien Diminutionszüchtung.
1 = Desoxygenisierungsvorlage mit $CrCl_2$-Lösung.
2 = Waschflasche mit H_2O.
3 = Impfkölbchen.
4 = Kolben mit Ausgangskultur
5 = Kolben für neue Kultur.
6 = O_2 - Messzelle (nach Tödt).
7 = Steriles Wattefilter.

und P-Transferierungsvermögen der Zellen nach jeder anaeroben Führung und nach jeder Lüftungsperiode geprüft wurden. Daraus ergaben sich abgestufte Entwicklungsreihen, die jeweils in Richtung der ab- und zunehmenden protoplasmatischen „Energieladung" verlaufen.

Im ersten Verfahrensgang, der energetischen Entladung, vermindert sich sukzessiv — parallel verlaufend mit dem Rückgang der Vermehrungsquote — die energetische Potenz der Zellen, anaerob Phosphat von aussen in sich aufzunehmen. Mit Eintritt der Anabiose, jenem Zustand latenten Lebens, welcher der vollkommenen Erschöpfung des anoxygenen Energiepotentials gleichkommt, ruhen alle anoxysynthetischen Zellfunktionen wie Proliferation, Phosphatwechsel, Glykogensynthese. — Im entgegengesetzten Vorgang, der energetischen Aufladung, werden die endergonisch inaktiven Anabiosezellen durch O_2-Einwirkung nach und nach reaktiviert, bis sie ihre ursprüngliche anoxygene Energiewirksamkeit zurückerlangt haben.

Zur Veranschaulichung des potentiellen Prinzips von Ent- und Aufladung der Gärungszellen, wie es bei allen aerob zuckerspaltenden Organismen mit graduellen Unterschieden symptomatisch ausgebildet ist (11), sind energetische Diminution und Restitu-

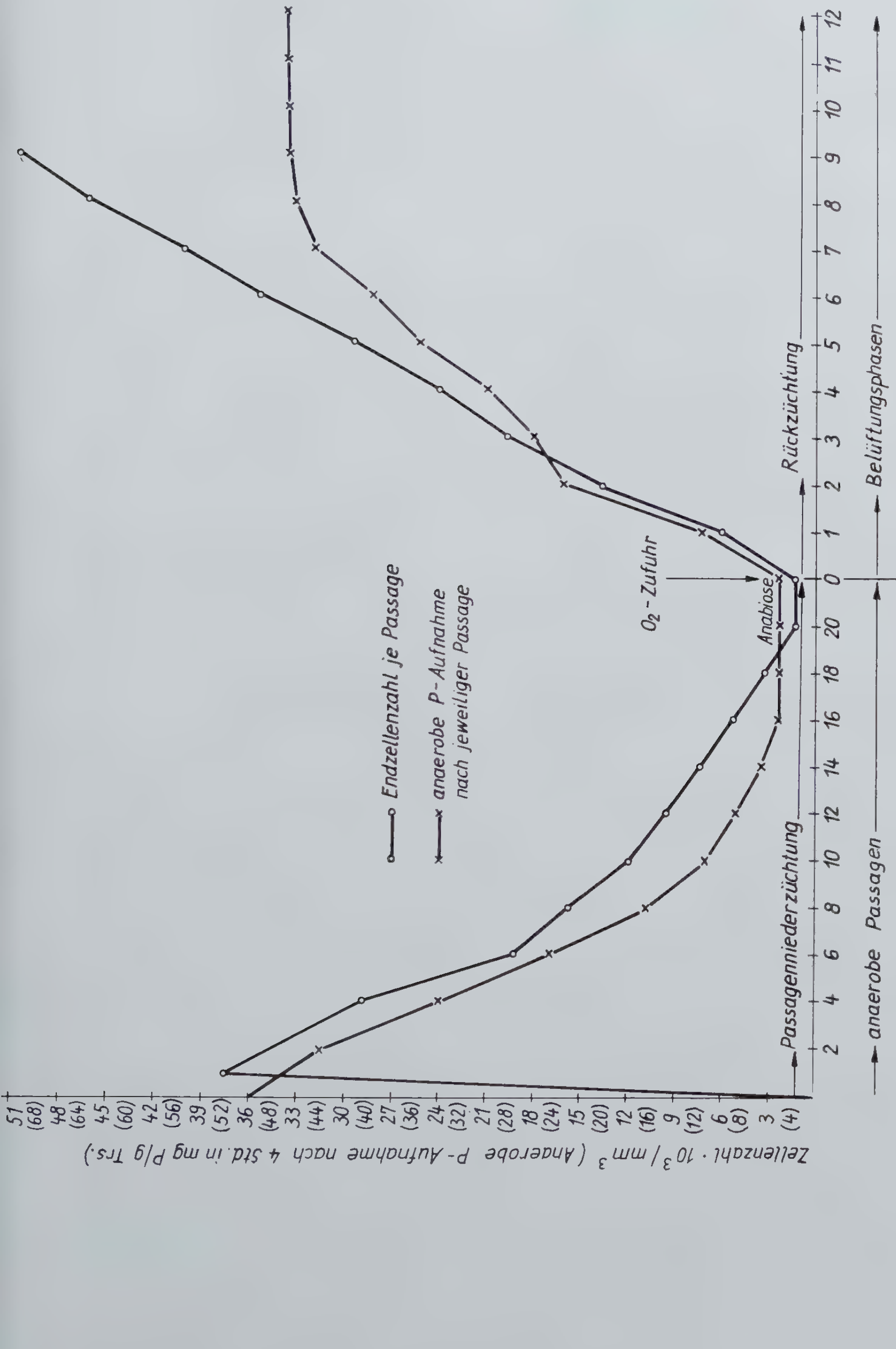


Abb. 4. Konversionsprinzip der energetischen Ent- und Aufladung von Gärungszellen (*Sacch. carlsbergensis* Frohberg).

tion am Beispiel von *Sacch. carlsbergensis* Frohberg graphisch gegenübergestellt (Abb. 4).

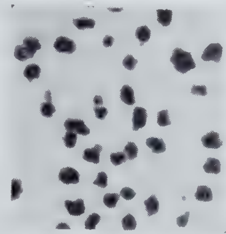
Die in der Zelle jeweils verfügbare Energie findet ihre relative Wertung in den anaeroben Proliferationsquoten und, parallel dazu, in den anaerob aufgenommenen P-Mengen. Abfall und Anstieg der beiden Synthesekurven entsprechen der energetischen Ent- und Aufladung der Zellen und geben demzufolge Aufschluß über die potentiellen Verhältnisse in jedem Reaktionsstadium. Aus dem Zusammenlaufen der Kurven im Anabiosepunkt wird der korrelative Schaltmechanismus erkennbar, der bei der anoxygenen Energieversorgung der Zelle regulativ fungiert.

Am eindrucksvollsten läßt sich das Konversionsprinzip der energetischen Ent- und Aufladung in Gärungszellen auf histochemische Weise unter Anwendung des bereits erwähnten M-T-Verfahrens demonstrieren. Aus dem Gesamtablauf können hier nur Ausschnitte als Schwarz-Weiss-Reproduktion von Originalfarbaufnahmen gezeigt werden. Im farbigen Original erscheinen die Zellphosphate als grüne bis sattgrüne Tinktionsstellen in Form von Punkten und Flecken, die sich im Schwarz-Weiss-Druck als dunkle Kontraste markant abheben. Eine ausführliche histochemische Dokumentation des Wechselspiels von energetischer In- und Reaktivierung haben wir an anderer Stelle gebracht (15).

Abb. 5 zeigt unter P-Mangel, aber sonst normal gezüchtete Gärungszellen, die bei anaerober P-Regenerierung stark überkompensierend Phosphat in sich aufnehmen (Abb. 6). Aus der folgenden Abb. 7, nach fortgeschrittener Diminutionszüchtung angefertigt, ist im Vergleich zu Abb. 6 ersichtlich, dass bei kontinuierlicher O_2-freier Passagenführung die Fähigkeit zur anaeroben P-Regenerierung immer mehr absinkt, bis schliesslich die M-T-Reaktion negativ ausfällt, also Anabiose eingetreten ist (Abb. 8). Umgekehrt gewinnen die P-verarmten Anabiosezellen (Abb. 8) unter O_2-Einwirkung ihr Vermögen zur anaeroben P-Transferierung nach und nach vollständig zurück. Im Verlauf fortgesetzter Belüftung tritt zunächst ein schwacher M-T-Effekt auf, der dann mittelstarkes Ausmaß annimmt (Abb. 9) und sich schliesslich bis zur typischen P-Überkompensation, hervorgerufen durch vorausgegangene P-Mangelzüchtung, intensiviert (Abb. 10).

In letzter Zeit konnte von uns festgestellt werden, dass sich endergonisch inaktive Anabiosezellen durch anaerobe Applikation von Dekokten bzw. Ätherextrakten aus Mikroorganismen, aus


5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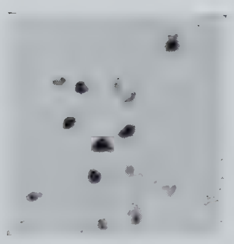
9

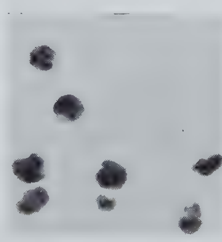
10

Abb. 5. Gärungszellen (*Sacch. carlsbergensis* Rasse U) im P-Mangelzustand; 600 : 1.

Abb. 6. P-arm gezüchtete Gärungszellen (*Sacch. carlsbergensis* Rasse U) nach anaerober P-Applikation; 600 : 1.

Abb. 7. P-arm gezüchtete Gärungszellen (*Sacch. carlsbergensis* Rasse U) im Stadium der fortgeschrittenen Diminutionskultivierung nach anaerober P-Applikation; 600 : 1.

Abb.8. P-arm gezüchtete Gärungszellen (*Sacch. carlsbergensis* Rasse U) im anabiotischen Zustand nach anaerober P-Applikation; 600 : 1.

Abb.9. P-arm gezüchtete anabiotische Gärungszellen (*Sacch. carlsbergensis* Rasse U) nach kurzer Belüftung, anschliessender 8tägiger anaerober Kultivierung und anaerober P-Applikation; 600 : 1.

Abb.10. P-arm gezüchtete anabiotische Gärungszellen (*Sacch. carlsbergensis* Rasse U) nach optimaler Belüftung, anschliessender 8tägiger anaerober Kultivierung und anaerober P-Applikation; 600 : 1.

Krebszellen aller Art sowie aus tierischen Organ- und Drüsengeweben in gleicher Weise wie durch O_2-Zufuhr energetisch aufladen lassen, so daß sie auch unter dieser bisher unbekannten Induktionswirkung wieder anoxygen zu proliferieren vermögen und überkompensierend Phosphat in sich aufnehmen, sofern sie P-arm vorgezüchtet waren (16, 17). Als "Agens" wurden von uns Lipoide und bestimmte höher ungesättigte Fettsäuren identifiziert, die als elektronenübertragende und energiefreisetzende Cofaktoren fungieren (18). In Tabelle 4 sind einzelne Testdaten angeführt, welche die Wirkungsfähigkeit von Zellextrakten und energieaktiven Metaboliten unter Beweis stellen.

TABELLE 4.

Anaerobe Restitution des anoxygenen Energiepotentials in anabiotischen Gärungszellen (*Sacch. carlsbergensis* Rasse U) durch anabiotisch aktive Extrakte bzw. isolierte Wirkstoffe.

Extrakte bzw. isolierte Wirkstoffe	Applizierte Menge/50 ml Nährsubstrat	Anaerobe Proliferation Zellenzahl/mm³		Anaerobe P-Transferierung (M-T-Reaktion)
		Anfang	Ende	
Dekokt aus *Sacch. carlsbergensis* Frohberg	15 ml	1300	20300	+ + +
Dekokt aus *Sacch. cerevisiae* Rasse XII	15 ml	800	18400	+ + +
Dekokt aus *Sacch. ellipsoideus* Winninger	15 ml	1000	12500	+ +
Dekokt aus *Pichia membranaefaciens*	15 ml	1300	20200	+ + +
Dekokt aus *Torulopsis* Stamm S	15 ml	1000	17000	+ +
Ergosterin aus *Sacch. carlsbergensis* Rasse U (puriss.)	50 mg	1100	22700	+ +
Squalen aus *Sacch. carlsbergensis* Rasse U (puriss.)	50 mg	900	19500	0
Nullprobe	(—)	800	800	—

+ + + = starke Reaktion, + + = mittelstarke Reaktion, + = schwach positive Reaktion, — = negative Reaktion, 0 = nicht angesetzt

Zur histochemischen Stützung der obigen Testdaten seien noch zwei kontrastierende Mikrobilder gebracht. Abb. 11 veranschaulicht, wie schon vorher Abb. 8, die im M-T-Test negativ reagierenden Anabiosezellen, während bei Einwirkung von Zellkochsaft aus *Sacch. cerevisiae* Rasse O, unter Fernhaltung jeder Spur von Sauerstoff, ein starker M-T-Effekt, phosphatische Überkompensation indizierend, in Erscheinung tritt (Abb. 12).

11 

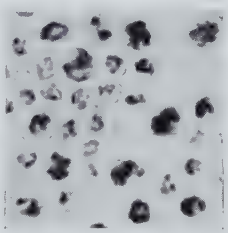 12

Abb. 11. P-arm gezüchtete anabiotische Gärungszellen (*Sacch. carlsbergensis* Rasse U) nach anaerober P-Applikation; 800 : 1.

Abb. 12. P-arm gezüchtete anabiotische Gärungszellen (*Sacch. carlsbergensis* Rasse U) nach 8tägiger anaerober Einwirkung von Kochextrakt aus *Sacch. cerevisiae* Rasse O und anaerober P-Applikation; 800 : 1.

Wenn man anfänglich noch annehmen konnte, dass durch den Prozess der Zuckerspaltung (Gärung) die Energie geliefert werde, welche die Gärungszellen dazu befähigt, in der Anoxybiose partiell zu proliferieren und anderweitige anoxygene Synthesereaktionen zu vollführen, so wird diese Annahme durch die Energiewirkung der O_2-freien Zellkochsäfte bzw. Lipoide widerlegt. Während die Anabiosezellen unvermindert gären, aber im Zustand der Zytostase nicht mehr zu proliferieren vermögen, was aus den Gär- und Proliferationswerten in Tabelle 5 hervorgeht, ruft Inkubierung mit O_2-freien Zellkochsäften oder Lipoiden anoxysynthetische Effekte hervor. Dieser lipoidgesteuerte, anoxergonisch gekoppelte Syntheseprozess, der nicht durch Gärung induziert werden kann (19), liefert den Beweis dafür, dass die beiden von allen Gärungszellen beherrschten Stoffwechselvorgänge — und zwar der anoxysynthetische, ausgelöst durch O_2-freie Zellkochsäfte bzw. Substanzen lipoider Art, und der asynthetische der Zuckerspaltung — in grundsätzlicher Weise voneinander verschieden sind (14).

TABELLE 5.

Sukzessive Diminution und Restitution des anoxygenen Energiepotentials von Gärungszellen (*Sacch. carlsbergensis* Stamm 26)

Züchtungsstadium (*Sacch. carlsbergensis* Stamm 26)	$Q^{N_2}_{CO_2}$	Anaerobe Proliferation Zellenzahl/mm³	P-Zunahme nach 4-stündiger anaerober P-Regenerierung in mg P/g Trs.
Normale Hefezellen	260	von 1 000 auf 55 000	40,0
Nach 2. anaerober Passage	260	,, 1 000 ,, 37 000	35,6
Nach 5. anaerober Passage	265	,, 1 000 ,, 25 000	22,1
Nach 10. anaerober Passage	265	,, 1 000 ,, 13 000	13,9
Nach 13. anaerober Passage	270	,, 1 000 ,, 7 000	7,0
Nach 20. anaerober Passage (Anabiose)	270	,, 1 000 ,, 1 000	0,0
Nach 1. Belüftungsphase	265	,, 1 000 ,, 11 000	12,5
Nach 2. Belüftungsphase	260	,, 11 000 ,, 25 000	23,6
Nach 3. Belüftungsphase	260	,, 25 000 ,, 36 000	32,3
Nach 4. Belüftungsphase	255	,, 36 000 ,, 43 000	39,7
Nach 5. Belüftungsphase	255	,, 43 000 ,, 56 000	41,2

Zusammenfassung.

An ausschliesslich atmenden Hefezellen konnte chemisch und zytologisch nachgewiesen werden, dass der Phosphatwechsel über die Zellmembran in gleicher Weise respiratorisch gekoppelt ist wie der Proliferationsakt. Im Gegensatz hierzu besitzen die Gärungshefen, als Vertreter des aerob zuckerspaltenden Stoffwechseltypus, die native Veranlagung, anoxysynthetische Zellreaktionen zu vollführen, so z.B. ohne Mitwirkung von Sauerstoff partiell zu proliferieren, Glykogen aufzubauen und Phosphat über die Zellmembran in sich aufzunehmen. Die Potenz zur Anoxysynthese herrscht bei ihnen jedoch nur so lange vor, wie sie über ein anoxygenes Energiepotential verfügen. Dieses erschöpft sich — einem Akkumu-

lator vergleichbar — im Verlauf fortgesetzter anaerober Passagenzüchtung mehr und mehr, bis im Endeffekt Anabiose eintritt. Umgekehrt erfolgt — bei erneuter und ausreichender Zufuhr von Sauerstoff — nach und nach seine vollständige Restitution, so daß die Zellen wieder wie vorher normal endergonisch, d.h. anoxysynthetisch leistungsfähig werden. Das sukzessive Geschehen der energetischen In- und Reaktivierung, in Wechselwirkung von Anoxy- und Oxybiose, lässt sich sowohl generativ als auch histochemisch verfolgen und demonstrieren. In analoger Weise wie Sauerstoff, gleichsam O_2-substituierend, wirken verschiedenartige Zell- und Gewebsextrakte bzw. bestimmte daraus isolierte Wirkstoffe; auch diese sind fähig, die anoxybiotisch induzierte Zytostase von Gärungszellen aufzuheben und anoxysynthetische Zellreaktionen wieder in Gang zu setzen.

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PHENOMENA IN MIXED CULTURES OF *SALMONELLA PARATYPHI* B; SEROLOGICAL CHANGES AND ADAPTATIONS OF THE BACTERIOPHAGE; INCOMPLETE NATURAL PHAGES; TRANSFORMATION OF PHAGE TYPES

by

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INTRODUCTION.

The natural phages of bacteria which could be demonstrated in the filtrate of a pure culture were called directly demonstrable natural phages in previous articles (SCHOLTENS 1950, 1952 and 1956). Not all phages are, however, found in this way; sometimes in a mixed culture of two strains bacteriophages were found which could not be demonstrated in pure cultures of the bacteria (FLU 1933, personal communication). These phages were called indirectly demonstrable (SCHOLTENS 1950, 1952, 1956). This phenomenon will be studied in the following article. It will be demonstrated that there is an exchange of genetic elements between the infecting phage and the prophage (or the prophage system) in the bacterium, and that various phage phenomena can be similarly explained. As these phenomena appear in the framework of the natural system of phage types of *S. paratyphi* B, this system will be briefly discussed here.

THE NATURAL SYSTEM OF PHAGE TYPES.

The sensitivity of a bacterial strain is influenced by the natural phages in the strains. Phage sensitivity and lysogenic properties probably depend on the same structures in the nuclear apparatus

TABLE 1. System of phage reacti

Group	Type	Gr				
		Adaptations of phage 1 of Felix and Callow				
		1	2	3a	Jersey	Bec Mep
M	3b-variation 1	—	—	—	—	—
	Midwoud	—	—	—	—	—
	B.A.O.R.	—	—	—	—	—
	M_6	—	—	—	—	—
S	"54"	—	—	(cl)	—	—
	"Sittard"	—	—	(cl)	—	—
	"18"	—	—	(cl)	—	—
	"87"	—	—	(cl)	—	—
A		—	—	—	—	—
J		—	—	(cl)	cl	c
B.M.		—	—	—	—	c
B		—	—	(cl)	—	—
2		—	cl	—	—	—
ungrouped types	1-most common	cl	cl	(cl)	cl	cl

cl = confluent lysis.
scl = semi confluent lysis.

Survey of the natural grouping of a part of the phage types *Salmonella paratyphi* B. There are two kinds of phage reactions, first the group reactions which all types of one and the same group of phage types have in common, and second the type reactions which differentiate the types in the groups. Group

of the strains. In most cases the bacteria are insensitive to the natural phages they carry. In the natural system of phage types of *S. paratyphi* B phage reactions and lysogenic properties have both been taken into account. All strains of the same type show not only the same phage reactions, but also the same lysogenic properties. In this natural system (Scholtens 1956, 1959) *S. paratyphi* B is subdivided into groups of types.

The types of the same group have phage reactions in common; parallel to this they have also lysogenic properties in common (table 1). Group M may serve as an example. All types of group M react with typing phages c, e and h; phage c lyses only types of this group. It is also of special interest that none of the types of

of *Salmonella paratyphi* B.

Phage reactions eactions with: Phages found in the mixed cultures						Type reactions with: Directly demonstrable type determining natural phages				Directly demonstrable type determining natural phages found in the types
e	d (Dundee)	c	f	h	b	I (3b)	IVb (Beccles)	IVa (Taunton)	VI	
cl	—	cl	—	cl	—	cl	cl	cl	cl	—
cl	—	cl	—	cl	—	—	cl	cl	cl	I
cl	—	cl	—	cl	—	(cl)	—	—	—	VI
cl	—	cl	—	cl	—	—	—	—	—	I, VI
cl	cl	—	cl	cl	+1	cl	cl	cl	cl	—
cl	cl	—	cl	cl	+1	—	cl	cl	cl	I
cl	cl	—	cl	cl	+1	cl	—	—	—	VI
cl	cl	—	cl	cl	+1	—	—	—	—	I, VI
—	cl	—	—	cl	—					
cl	cl	—	—	scl	cl					
cl	cl	—	—	—	cl					
cl	cl	—	—	cl	cl					
cl	cl	—	cl	cl	—					
cl	cl	—	cl	cl	cl					

(cl) = the reaction is not always, or even rarely present.
+ 1, + 2, + 3 increasing number of plaques.

reactions give: 1. the adaptations of phage 1 of FELIX and CALLOW and 2. bacteriophages found in the mixed cultures. Type reactions give the directly demonstrable, type determining natural phages I, IVa, IVb, and VI.

group M react with typing phage d which lyses types of all other groups. Parallel to this, strains of all types of group M have a lysogenic property in common. All produce a natural phage of lytic spectrum d. This phage may be found in pure cultures of the strains (see below). In most cases, however, it is found only in mixed cultures with strains of types of other groups. In a similar way strains of group S give the same group reactions and all strains of group S produce phages lytic spectrum c in mixed cultures with strains of types of group M. These phenomena will be analysed in this paper.

The groups are subdivided into types. These types are characterised by an increasing content in natural phages I, IV and VI

and by a parallel decrease in sensitivity to these phages (table 1). Again group M may serve as an example.

The types of group M are distinguished with natural phages I, IVa and IVb and VI. Here also there is a parallelism between phage reactions and lysogenic properties. Type 3b variation 1 produces none of these three natural phages and is sensitive to all; type Midwoud produces phage I and is resistent to this. Type B.A.O.R. produces phage VI and is similarly insensitive to IV and VI. Type M_6, discovered by RISCHE in 1959, produces I and VI, and is similarly insensitive to I, IV and VI. Moreover this strain contained phage dII.

A similar increase in the content of natural phage and a parallel decrease in phage sensitivity is to be seen in group S. All types of group S react with phages d, e and f and h. Type "54" contains none of phages I, IV and VI and is sensitive to these; type "Sittard" differs from type "54" in that it contains phage I and is resistant to phage I; type "18" differs from type "54" in that it contains phage VI and is resistant to phages IV and VI; type "87" differs from type "54" in that it contains phages I and VI and is accordingly resistant to phages I, IV and VI. These types differ among themselves in the same manner as types of group M.

TECHNIQUE AND NOMENCLATURE.

1. Phage reactions. The technique of CRAIGIE and FELIX (1947) and of FELIX and CALLOW (1951) was followed.
2. Serological reactions. The technique which was described in a previous article (SCHOLTENS 1955) was followed.
3. Mixed cultures. A drop of fresh broth culture from each strain was inoculated in 100 ml of broth and incubated at 37° C.
4. Research on the presence of phages c and d. Phage c can multiply on type B.A.O.R., phage d on strains of type "Dundee". One ml of the preparation to be examined for phages c and d is put in broth which has been inoculated with one of the above strains. Then the preparation obtained is tested on strains of types B.A.O.R., Dundee, "87", 3a variation 3, 3aI Leeuwarden and Beccles-Meppel, in other words, of types which are as much as possible resistant to the directly demonstrable natural phages (the type Beccles-Meppel is the only type found of group B.M.). Interfering phages which are often present, as will appear from the following, have often (though not always)

a lower concentration than phages c or d. The phages can be obtained in pure culture on strain *S. java* 13217.

5. Nomenclature. Several phages exist of lytic spectrum c differing serologically among each other. When a property of phages c is referred to, a property is meant which all these phages have in common. Phages II and VI are natural directly demonstrable phages of *S. paratyphi* B which possess a definite serology. This serology, if present in a different phage, is designated by the figure II or the figure VI. Thus phage c VI is a phage of lytic spectrum c and serology VI.

Results.

The occurrence of the natural phages c and d in pure cultures and in mixed cultures.

The occurrence of the phages c and d in mixed cultures was studied with strains of groups M and S of the natural system of phage types. Mixed cultures were made of each of three strains of type Midwoud (Pty 6529, Pty 1108 and Pty 821) with each of three strains of type Sittard (Pty 3943, Pty 572 and Pty 1165). In most of these mixed cultures the phages c and d were found after a week's incubation. An examination was made to establish whether the phages c and d could be found in the filtrates of pure cultures of each of the strains.

Twenty-four hours and 14×24 hours old broth cultures of each of the strains were filtered, and each of the strains was grown in the filtrate of each of the strains of the other type. All the cultures were tested for the presence of the phages c and d after 24 hours and after 14×24 hours. The phages c and d, that are so easily found in the mixed cultures, were found once only in a 14×24 hours' old broth culture. The results of the experiments can be seen in table 2. The fact that these natural phages are demonstrable only with difficulty or not at all in the filtrates of pure cultures of the strains stands in sharp contrast to the behaviour of other natural phages such as phages I, IV and VI. One only needs to filtrate a broth culture of the strains and to add the filtrate to a sensitive strain in order to find these phages a few hours later.

It was ascertained in a subsequent experiment how long it took for the phages mentioned to occur in the mixed cultures. For this purpose, mixed cultures consisting of each of nine strains of different types of group M together with a strain Pty 3943 of type Sittard

TABLE 2.

Survey of the not directly demonstrable phages found in an experiment in mixed cultures Midwoud/Sittard and in cultures of strains of the one type in filtrates of 24 hours broth cultures of the other type.

	Type Midwoud					
	Strains			Filtrates		
	Pty 6529	Pty 1108	Pty 821	Pty 6529	Pty 1108	Pty 821
Type Sittard						
Strains						
Pty 3943	d	c, d	d	—	—	—
Pty 572	d	c, d	c, d	—	—	—
Pty 1165	d	c, d	c, d	—	—	—
Filtrates of strains						
Pty 3943	—	—	—			
Pty 572	—	—	—			
Pty 1165	—	—	—			

The strains and filtrates indicated in the vertical column are inoculated with the strains and filtrates indicated in the horizontal row.

were made in bottles of broth. Every day a sample of a few ml was taken out and shaken with chloroform. This sample was tested for the presence of phages c and d. In general, it appeared that phage d could be demonstrated somewhat earlier than phage c and that phages c and d are produced later in mixed cultures of strain Pty 3943 with strains of type B.A.O.R. than in mixed cultures of strain Pty 3943 with strains of types 3b variation 1 and Midwoud. This result can be seen in table 3.

TABLE 3.

Survey of the length of time which is necessary for the appearance of phages c and d in the mixed cultures of the indicated strains with strain Pty 3943, type Sittard.

Strains							
Type 3b variation 1		Type Midwoud			Type B.A.O.R.		
4096		1108	6529	821	4434	4493	1117
Number of days after which phages c and d occur in mixed cultures							
c	d	c d	c d	c d	c d	c d	c d
3	2	3 1	3 1	4 2	1 >10	6 6	5 5

Summarising, we may say that these two phages are found in mixed cultures of strains that neither liberate phage c nor d in pure cultures after a certain lapse of time.

The occurrence of phages c and d in pure cultures of some strains, and in cultures of strains in the filtrates of other strains.

Different results were obtained with a few strains of group M and a few strains of group S. A phage of lytic spectrum d (lytic spectrum "Dundee" of Felix and Callow) could be directly demonstrated in the filtrate of strain B 2553 of Felix and Callow and in the filtrates of all strains of type Midwoud found in a certain epidemic and in strain W 4563/58 of type M_6.

When strains of type Sittard are grown in filtrates of these strains (*i.e.* in filtrates containing phage d), they produce a phage of lytic spectrum c which they do not produce in pure cultures. The phage d in the filtrates causes the strain of type Sittard to liberate phage c. Phages c and d are thus found in these preparations.

A parallel phenomenon could be shown to occur with strain

Pty 3554, type "54" of group S. A phage was found in the filtrate of this strain which lysed a strain 13217 of *S. java*. This natural phage does not attack strains of type Midwoud. If strains of type Midwoud (which do not liberate phage d in pure cultures) are grown in filtrates of strain Pty 3554, this natural phage acquires lytic spectrum c and the strains of type Midwoud liberate phage d.

If grown in the presence of given phages, the strains studied in the foregoing paragraph liberate phages they do not liberate in pure cultures.

In the filtrates of strains of type 1, type Jersey, type Beccles-Meppel and type 3b also phages are found that may acquire lytic spectrum c and the propensity to cause the strains of type Midwoud to liberate phage d.

Adaptation of the natural phage from strain Pty 3554; change of serology during the adaptation.

Strain Pty 3554 of type "54" from group S contains none of the natural phages I, IV or VI. A bacteriophage can, however, be demonstrated in the filtrate of a pure culture of this strain in broth with the aid of strain *S. java* 13217. This phage produces small pin point plaques and is neutralised by none of the serums anti I, IV or VI, but is by serums anti phage II. This phage will be called phage II S further on. When this phage was propagated on strains of types of group M, it changed its lytic spectrum and acquired the lytic spectrum c. It will be shown here that this change of virulence is an adaptation. Phages isolated from a separate plaque on strain *S. java* 13217 were titrated on strains of all types of *S. paratyphi* B. The preparation gave confluent lysis on strain *S. java* 13217 up to a dilution of 1/100; undiluted it gave a few microplaques on strains of group M; there was no reaction in higher dilutions. On strains of other types there was no reaction at all. When, however, passages were made with this phage on strains of type Midwoud this phage acquired a virulence to strains of group M. Now these phages attack these strains in an equally high titre as strain *S. java* 13217, but none of the types of the other groups. The phage has obtained lytic spectrum c (table 1). If we assume that an adaptation of a phage is a modified form that by multiplication on a given strain has obtained an increase of titre for this strain in comparison with the titre as ascertained upon other strains, then we must consider this phage of lytic spectrum c as an adaptation of the latent phage from strain

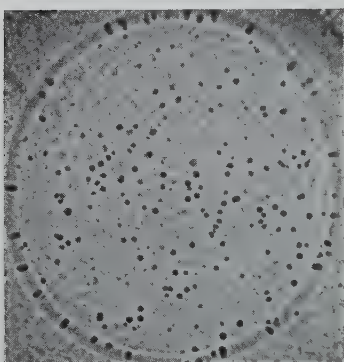
TABLE 4.

The titration of phage II S, the natural phage from strain Pty 3554, type "54", multiplied on strain *S. java* 13217, before and after a series of passages on strain Pty 6529, type Midwoud. The phage has acquired lytic spectrum c.

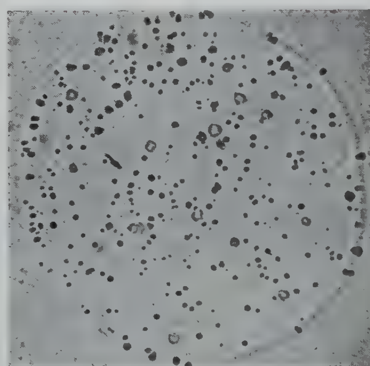
Titrated on strains	Phage II S before passages on type Midwoud				Phage II S after passages on type Midwoud			
	undil.	10^{-1}	10^{-2}	10^{-3}	undil.	10^{-1}	10^{-2}	10^{-3}
Salmonella java	cl	cl	cl	+1	cl	cl	cl	+3
B 2553, 3b variation 1	+ 2 μ μ	—	—	—	cl	cl	cl	+3
Pty B 6529, Midwoud	+ 3 μ μ	—	—	—	cl	cl	cl	+3
Pty B 1117, B.A.O.R.	+ 2 μ μ	—	—	—	cl	cl	cl	+3
strains of further types	—	—	—		—	—		—

Pty 3554. If the adapted phage is now retransferred to strain *S. java* 13217 the change in lytic spectrum remains. In a similar way phage II S can be adapted to the other types of group M, types 3b variation 1 and B.A.O.R. whereby it also acquires lytic spectrum c. A serological change observed in the phage during the adaptation upon type B.A.O.R. throws light upon the way this and other adaptations are brought about.

The phage of lytic spectrum c obtained by an adaptation of phage II S on type Midwoud or on type 3b variation 1 has small plaques and serology II just like the original phage. If, however, the phage II S is adapted to a strain of type B.A.O.R. quite different results are obtained. On multiplication on strains of this type phage II S shows not only an increase in virulence but also a greater variability in plaque form. Larger plaques are observed also. These can be seen in fig. 1.



Plaques of phage c II.



Plaques of phages c II and c VI.

Fig. 1.

On subculture from these larger plaques phage preparations are obtained, the particles of which are inactivated by a serum anti phage VI, a serum obtained with the natural phage VI of the bacterium on which the infecting phage multiplies. The phage II S, from which these preparations originated, does not react at all with this serum. The phage acquires the serology of the natural phages of the bacteria whereupon it is propagated. The inactivation by serums anti phage VI and II of the isolated serologically changed phages was given in table 2 of a paper published before (Scholtens

1952). These phages are inactivated by serums anti VI in and up to the titre limit and by serums anti II only in stronger concentrations. The serological difference between phages of large plaques and phages of small plaques can also be directly demonstrated in the mixtures of phages obtained; only the large plaques disappear with serum anti-phage VI; with serum anti-phage II in higher titres only the small ones. This change in serology is neither a phenomenon observed only when a peculiar phage is propagated upon a peculiar strain, nor is it restricted to the serology of the natural phage VI. The same change in plaqueform and serology occurs in the phage dII when it is propagated upon strains of type Dundee or type "87", that contain the natural phage VI, and is not seen when this phage is propagated upon types from the same groups that do not contain this natural phage. Phage dII may also take on the serology IV. This occurs when the phage dII is propagated upon strains of type Taunton. Phage dIV is found after a certain number of passages.

Summarising, we may say that, when the bacteriophage of serology II from strain Pty 3554 is transferred to strains of type B.A.O.R., it increases its virulence for this strain and changes serologically. It will contain elements from the natural phage VI of this strain, or in other words, elements from or formed under the influence of the prophage system of the infected bacteria.

At the Congress for Microbiology in Copenhagen Felix (1947) put forward the theory that the changes in the bacteriophage which lead to an increase in virulence consisted of an incorporation of material from the bacterium. Although it was not proved here that the increase in virulence was induced in this way, it was, indeed, demonstrated that the increase in virulence and incorporation of material from the bacterium both take place in such a way that it is logical to explain the one by the other.

Influence of the infecting phage on the release of the natural phage.

The change in serology is, however, not the only phenomenon to be observed with the adaptation of the bacteriophage. A series of passages on strains of types of group M was made with the phage from strain Pty 3554. The preparation obtained after each passage was not only examined for the presence of phage c, but also for that of phage d. It appeared that after a few passages on type Midwoud phage d occurred in the preparation in addition to phage c. It could

be demonstrated that this phage was liberated by the bacterium under the influence of the adapted phage c.

When preparations which contain phage c only are allowed to act on strains of group M, phages c as well as d can always be demonstrated in the preparations thus obtained. Phage d can be eliminated from the preparations which contain phages c and d in two ways; firstly, by diluting the preparation since phage c is present in an higher titre than phage d, and secondly, by growing the strain in a pure culture on *S. java*, strain 13217.

A series of tenfold dilutions was made of a preparation phage c obtained by transferring phage II S on strain Pty 1117 (type B.A.O.R.). One ml of each of these dilutions was put into each of two test tubes with broth so that two series were obtained with decreasing quantities of phage. To one series, strain Pty 1117 was added by which phage c could be demonstrated; to the second series, strain Pty 147 (type Dundee) was added on which phage of lytic spectrum d can multiply. After 24 hours incubation the preparation was tested on the strain added. It appeared that phage c was found in a titre of 10^{-5} whereas phage d was found only in a titre of 10^{-2}.

In higher dilutions, therefore, only phage c is found. Larger amounts of dilutions 10^{-5}, 10^{-6} and 10^{-7} were made and 1 ml of these dilutions was added to each of 20 test tubes. Ten of the test tubes were inoculated with strain Pty 1117, type B.A.O.R. and ten of them with strain *S. java* 13217. Phage c multiplies on the first strain and phage c as well as phage d multiplies on the latter. After 24 hours incubation all tubes were examined for phages c and d by testing immediately and after multiplying as described above. In some tubes containing strain *S. java* 13217 phage c was found. Phage d was never found. As this strain is sensitive to both phages this can be considered as a check on the presence of phage d in the preparation examined. In the tubes where strain Pty 1117 was inoculated either no phage was found or phage c was found as well as phage d. Phage c was demonstrated with approximately equal frequency, with both strains. A correlation thus exists between the appearance of phage d and the meeting of phage c with strain Pty 1117 (B.A.O.R.) in the culture. This correlation can be seen in table 5. This can be explained by assuming that phage c stimulates strain 1117 (B.A.O.R.) into giving off its own natural phage. A hypothesis by way of explanation is that (pro) phage d is present incompletely in the strains of types of group M and is completed by

TABLE 5.

Correlation between strain type B.A.O.R. being infected with phage c and the production of natural phage d of the bacterium.

Dilution of the phage preparation	Number of samples of 1 ml examined	Inoculated with strain	Number of times natural phages c and d were found				
			neither c nor d	only c	only d	c and d at once	c and d after a further passage on strain Pty 1117
$1/10^5$	10	Pty 1117, BAOR	1	—	—	5	4
$1/10^6$	10	Pty 1117, BAOR	6	—	—	3	1
$1/10^7$	10	Pty 1117, BAOR	9	—	—	—	1
$1/10^5$	10	*S. java* 13217	—	10	—	—	—
$1/10^6$	10	*S. java* 13217	6	4	—	—	—
$1/10^7$	10	*S. java* 13217	10	0	—	—	—

Dilutions were made of a phage preparation and each time ten samples of 1 ml of the higher dilutions were inoculated with strain Pty 1117 (BAOR) and ten samples of 1 ml were inoculated with *S. java* 13217. The samples inoculated with Pty 1117 (BAOR) in which phage c was found contain phage d also. The samples inoculated with *S. java* contain phage c only, or no phage at all, although both phages can multiply in each other's presence.

the introduction of genetic material from phage c. Further arguments will be put forward in support of this point of view.

A second method of separating phage d from phage c is by growing this phage in a pure culture on strain Pty 13217 of *S. java*. Passages on this strain do not in general change the behaviour of phages towards *S. paratyphi* B. When a phage c, which has been grown pure in this manner, is allowed to act on strains of group M, phages c and d are found in the preparations, though phage d is not liberated by the strains in pure culture.

Transmission of a serological property of an infecting phage to a natural phage of the bacterium.

It was demonstrated in a previous paragraph that phage c takes over the serology VI after passages on strains which possess natural phage VI. When now phage c VI is obtained in a pure culture on strain *S. java* 13217 and is grown on strains of type Midwoud (which do not contain the natural phage VI) not only phage d II but also phage d VI is found in addition to phage c VI in the preparation obtained (When phage c II acts on these strains they produce only phages d II). Serological property VI is now taken over by the infecting phage c VI to natural phage d in the infected bacterium. Genetic material from the infecting phage is thus taken over to natural phage d in the infected bacterium. This lends support to the hypothesis that natural phage d which is incompletely present in this bacterium is completed by the introduction of material from the infecting phage. Phages which are only found in mixed cultures can thus be called incomplete prophages. The above mentioned experiments were carried out as follows.

Phage c VI was obtained from the natural phage II S of strain Pty 3554 as follows. A separate plaque obtained with the filtrate of this strain on strain *S. java* 13217 was subcultured. This phage was adapted on strain Pty 1117 (B.A.O.R.) by some ten passages. From the filtrate phage c VI was obtained in pure culture on strain *S. java* 13217. This phage c VI was added to broth inoculated with strains Pty 6529[1]) and B 2553 (type 3b variation 1) respectively. It was filtered off 24 hours later. On immediate testing of the filtrate on strain Pty 572 (type Sittard) large plaques were seen which on subculture produced preparations of phage d VI. The serological property VI is here therefore carried over from the infecting phage c

[1]) type Midwoud.

VI to natural phage d which in strain Pty 6529 is incompletely available and in strain B 2553 can be directly demonstrated (see above).

One can now reverse the role of the infecting phage and the natural phage and allow phage d to act as infecting phage on strains of type "54" or upon other strains that produce a phage from which phage c may be obtained by adaptation. Phage d is allowed to make a number of passages upon such a strain and is obtained pure by subculture from a single plaque on strain *S. java* 13217. Now it is allowed to grow on strain 3554 for example. Of course phage d is found in the preparation, but phage c is also found. Instead of the phage of low virulence S II, the strains have liberated a phage c that otherwise is obtained from phage S II by adaptation. It must be assumed here that the prophage in the bacterium has absorbed material from the infecting phage and in doing so phages are released in an adapted form. The adaptation takes place, therefore, in two different ways; firstly, by the phage in one bacterium forcing its way into a second bacterium and absorbing material from the prophage in this bacterium; secondly, by the natural phage of this bacterium forcing its way into the first bacterium (which separates the unadapted phage) and yields to this unadapted phage material by which it is released in the adapted form. If the experiment is performed with a phage d VI phages c VI may be found. This is a further argument for the hypothesis put forward above.

Transformation of phage types.

A transformation of phage types was obtained in the following way. A filtrate which contained the adapted phages c and d was obtained by growing strains Pty 6529 and Pty 1108 (type Midwoud) in the filtrate of strain Pty 3554 type "54" and filtering off a week later. A broth culture of strains Pty 6529 and Pty 1108 diluted to $1/10^6$ was added to 9 ml of this filtrate and shaken; 15 minutes later 0.3 ml of this fluid was plated. Nearly all colonies showed irregular shapes and deviations, which betrayed infection by the bacteriophage (fig. 2).

These colonies were subcultured in broth and incubated for 24 hours. On typing, these cultures gave no phage reactions at all with the natural system (or only reactions with phages $0_{1.2.3}$ of Felix and Callow). They produced phage c as well as phage d. When these broth cultures, however, were plated there were mostly normal colonies and a few with freakish shapes as in fig. 2. The normal

colonies yielded mainly cultures which completely agreed with the original "Midwoud" strain and a few cultures which produced phage c and were resistant to these. In all, 192 colonies were examined.

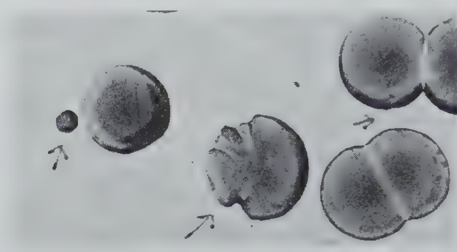


Fig. 2. Phage infected colonies.

The irregularly shaped colonies again yielded cultures which gave no phage reactions, but plated returned normal colonies and some freaks. Nearly all these normal colonies now appeared to produce phage c and to be resistant towards it, but for the rest they agreed with type "Midwoud".

Some cultures, however, were found which behaved irregularly. First, strains were found which were resistant to phage c, and which additionally were sensitive to phages d and f and to adaptation 3a-3aI of phage I of Felix and Callow. These strains now might have been diagnosed as type "Sittard". Furthermore variants were isolated which had retained a sensitivity to phage c, but had acquired additionally the sensitivities mentioned above. These changes in phage sensitivities are recorded in table 6; only changes in group reactions are dealt with. The type reactions have been omitted from the table. These transformations of phage types will be discussed elsewhere in greater detail.

Discussion.

The phenomenon that natural phages are only released in mixed cultures and are not found separately in pure cultures of the strain can be explained by the prophages occurring incompletely in the bacterium and being completed by material from an infecting phage. However, the observation must be made that, if also the prophage is incomplete in both strains, natural phages have been found in

TABLE 6.

Group reactions of new types originating from type Midwoud under the influence of the phage from type "54".

Type	Group reactions											Directly demonstrable natural phages	
	Adaptations of phage 1 of Felix and Callow					Lytic spectrum of phages found in mixed cultures							
	1	2	3a-3aI	Jersey	Beccles Meppel	e	d	c	f	h	b		
Midwoud	—	—	—	—	—	cl	—	cl	—	cl	—	I	
"54"	—	—	cl	—	—	cl	cl	—	cl	cl	—		II S
Modified types obtained	—	—	—	—	—	cl	—	—	—	cl	—	I	cII
from Midwoud influenced	—	—	cl	—	—	cl	cl	—	cl	cl	—	I	cII
by phage c II	—	—	cl	—	—	cl	cl	cl	cl	cl	cl	I	

mixed cultures. These phages are composed of elements derived from both strains, as described earlier (Scholtens, 1952). The existence of incomplete or defective prophages was reported for *Pseudomonas aeruginosa* by Jacob (1950), for *Bac. megatherium* by Lwoff and Siminovitch (1951), and for *E. coli* by Lederberg and Lederberg (1953) and Appleyard (1954).

The exchange of genetic material between the infecting phage and the natural phage was described earlier by Bertani (1953-1954). Here the infecting phage was a variant of the natural phage.

It is obvious that the concept of an adaptation being brought about by the incorporation of material from the prophage in the bacterium is on the same lines as the concept of Luria and Herman (1952), Bertani and Weigle (1953) and Weigle and Bertani (1953) that the changes in lytic spectrum are host induced. Anderson and Fraser (1956) also agree that part of the adaptations of Vi phage II are host induced. The phage does not multiply itself, but is multiplied by the cell it enters so that with each change in the phage the host may play a part.

It is not sufficient for a bacterium to have a substance on its upper surface specifically absorbing the bacteriophage to be sensitive to this bacteriophage. The bacteriophage must be able to react with certain structures in the bacterium. It was demonstrated by Craigie (1946), Anderson (1951), Nicolle and Hamon (1951) and by Anderson and Felix (1953), and Felix and Anderson (1951) that the prophage present in the bacterium influences the phage resistance of the bacteria. It was here demonstrated that the bacterium reacts with the prophage in the bacterium and that the prophage can be incompletely present in the bacterium. It is possible that the structures mentioned above with which the bacteriophage must be able to react form the prophage system itself or can be considered as an incomplete prophage system.

Summary.

The phenomenon that natural phages are only released in mixed cultures and are not found in pure cultures of bacterial strains has been discussed.

It was described how an infecting phage absorbs material from the natural phage in the bacterium and the other way round, that the natural phage absorbs material from the infecting phage. Hereby

the bacteriophages can change serologically. Simultaneously the bacteriophage can be adapted. The phenomenon that natural phages are only released in mixed cultures is in some cases explained by assuming that the prophage is incompletely present in the bacterium and is completed by material from an infecting phage.

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THE WINE YEASTS OF THE CAPE

PART IV. — ASCOSPORE FORMATION IN THE GENUS *BRETTANOMYCES* [1])

by

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(Received March 11, 1960).

Introduction.

The genus *Brettanomyces* (*in sensu* Lodder et Kreger-van Rij) has been accepted to be anascosporogenous by most zymologists [Claussen (1904), Kufferath and van Laer (1921), Custers (1940), Diddens and Lodder (1942), Skinner (1947), Shimwell (1947), Mrak and Phaff (1948), Wickerham (1952), Lodder and Kreger-van Rij (1952), Peynaud and Domercq (1956), Lodder, Slooff and Kreger-van Rij (1958), van der Walt and van Kerken (1958, 1959)].

Agostini (1950), however, studying a number of incompletely determined *Brettanomyces* species isolated from wines, reported the observation of ascospore formation during a rather complex life-cycle claimed for the species. Haploid cells, it was maintained, conjugated to form a diploid generation which, in turn, conjugated to form tetraploids. Triploid individuals were observed to arise from the conjugation of haploid and diploid individuals. It was further claimed that polyploid cells arose from the fusion of chains of haploid cells. The ploidy of the cells was established by their "chondriosome count". Diploid, triploid, tetraploid and even polyploid cells were directly transformed into asci which formed 1–3 oval ascospores. The spores were, however, neither stained nor isolated, nor was any attempt made to demonstrate their heat resistance. In view of this,

[1]) Part I, II and III: Antonie van Leeuwenhoek **24**, 239, 1958; **25**, 145, 1959; **25**, 449, 1959.

their identification as true ascospores seems to be open to some doubt.

On the other hand, during the current study of the distribution of the genus *Brettanomyces* in South African wineries, several freshly isolated strains of *Brett. intermedius* and *Brett. schanderlii* were obtained which sporulated actively. On suitable media, 1–4 hat-shaped ascospores were formed. These stained readily with the common ascospore stains and proved to be more heat-resistant than the vegetative cells. Reinvestigation of strains of *Brett. schanderlii*, as well as several strains of *Brett. bruxellensis*, held by the Centraal Bureau voor Schimmelcultures subsequently revealed that these, too, were ascogenous. Ascospore formation could, however, not be detected in the strains of *Brett. anomalus* or *Brett. claussenii* which were available to the authors.

Experimental.

Cultures:

Brett. intermedius
Strain B 30–b — isolated from cellar equipment
Strain M51 — isolated from cellar equipment
Strain V61 — isolated from a bottled dry white wine
Brett. schanderlii
Strain CBS–2796
Strain CBS–2797
Strain H5 — isolated from a drain in a winery
Strain M42 — isolated from a drain in a winery
Brett. bruxellensis
Strain CBS–74
Strain CBS–96
Strain CBS–97
Brett. anomalus
Strain CBS–77
Strain CBS–3026
Brett. claussenii
Strain CBS–76
Strain CBS–1938
Strain N18 — isolated from cellar equipment

Results.

The cultures of *Brett. intermedius*, *Brett. schanderlii* and *Brett. bruxellensis* sporulated readily at 25° C. on Wickerham's stock culture agar, yeast water agar and Fowell's acetate agar, provided that these media were adequately supplemented with vitamins. Ten ml of a stock vitamin solution (containing 200 γ biotin, 40 mg

calcium pantothenate, 200 γ folic acid, 200 mg inositol, 40 mg niacin, 20 mg *p*-aminobenzoic acid, 40 mg pyridoxine hydrochloride, 20 mg riboflavin and 100 mg thiamine hydrochloride per litre) was added per litre medium after sterilization. *Brett. intermedius* and *Brett. schanderlii* sporulated after 9–14 days, while *Brett. bruxellensis* sporulated after 6–9 days.

Conjugation was not observed to precede ascus formation, diploid cells apparently being directly transformed into asci. In the case of *Brett. intermedius* and *Brett. schanderlii*, the mature ascospores were plainly hat-shaped. In the case of *Brett. bruxellensis* the mature spores were generally spheroidal with nearly tangential rings, sometimes resembling spores of the Saturn type. Mature asci disintegrate rather soon, liberating the spores which tend to agglutinate. The mature ascospores stain selectively with the modified Schaeffer-Fulton malachite green stain, as well as with Mann's eosin-methyl blue. Mature ascospores are alcohol-acid fast after staining with Ziehl Neelsen's carbol fuchsin.

The ascospores germinate relatively soon if Wickerham's stock agar is used to observe sporulation.

Vegetative and sporulating cultures of *Brett. bruxellensis* CBS-97, *Brett. intermedius* M51 and *Brett. schanderlii* CBS-2796 were heat-treated at 57° C. according to the technique employed by Wickerham and Burton (1954) for the isolation of heterothallic mating types and actively sporulating cultures. Sporulating cultures all survived heating to 57° C. for 2-4 minutes longer than the time required to inactivate vegetative cells. Ten to fourteen days were usually required for the adequate development of colonies formed by the heat-treated ascospores. Diploidization was observed, as well as the budding of zygotes. Vegetative cells were directly transformed into asci. No evidence was found to support the complex life-cycle involving the formation of asci from triploid, tetraploid or polyploid generations, as reported by Agostini (1950). All three strains were homothallic.

To date no ascospore formation has been observed with the strains of *Brett. anomalus* and *Brett. claussenii* studied.

Discussion.

The reason why ascospore formation has not previously been observed in this genus would seem to be due to the fact that previous investigators had always studied cultures on media of inadequate

vitamin content. While it must be admitted that starvation of cells by restricting the available sources of carbon and or nitrogen in sporulation media (*e.g.*, gypsum blocks, tap-water agar, Gorodkowa agar and Fowell's agar) generally favours sporulation, it appears that sporulation nevertheless requires adequate amounts of growth factors. For most ascosporogenous yeasts these requirements are met by using large transfers of vigorous and well-nourished cells. In the case of the extremely fastidious *Brettanomyces* species, where the organisms require much higher concentrations of growth factors, particularly biotin and thiamine, for good growth, large transfers of cells to the sporulating medium contain inadequate amounts of vitamins. It therefore seems advisable to introduce the use of vitamin-enriched sporulation media, particularly for such species as have hitherto been regarded as anascosporogenous.

The discovery of ascus formation in three species of *Brettanomyces* necessitates their reclassification. There are two possible ways of doing this. Firstly, it might be maintained that some effort should be made to include these species in the existing genera of the *Endomycetaceae*. The nitrate-positive species would then have to be assigned to the genus *Hansenula*, while the nitrate-negative species would be assigned to the genus *Endomycopsis* (*in sensu* Wickerham). On the other hand, as the type species of the genus, *Brett. bruxellensis*, is ascosporogenous, it would also be possible to regard the entire genus as ascosporogenous and transfer it as a whole to the *Endomycetaceae*. Admittedly, ascospore formation has not as yet been observed in *Brett. anomalus* or *Brett. claussenii*. This may either be due to the fact that the extant strains examined have lost their ability to sporulate by their long maintenance in collections, or that they exist as haploid heterothallic mating types, of which insufficient strains exist for fruitful matings. However, this transfer requires the redefinition of the genus in such a way as will eventually permit the construction of a phylogenetic line. This the authors do not propose to attempt at the moment, since many of the properties of the species of this genus, particularly the biochemical characteristics, are as yet incompletely understood and are in want of further study.

Studies on this genus are being continued.

Summary.

Ascospore formation has been observed in three species of the

genus *Brettanomyces, viz. Brett. bruxellensis, Brett. intermedius* and *Brett. schanderlii*. On media of adequate vitamin content these species form 1–4 hat-shaped ascospores. The spores are liberated rather soon after maturation. Heat treatment of ascogenous cultures indicated that these species are homothallic.

In view of the fact that ascospore formation has been observed in the type species, *Brett. bruxellensis*, the transfer of the genus to the *Endomycetaceae* should be considered.

Acknowledgements.

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ISOLATION OF NON-TOXIGENIC DIPHTHERIA BACTERIA AND PHAGES DURING A LOCAL OUTBREAK OF DIPHTHERIA

by

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(Received December 3, 1959).

A new light has been thrown on the possible significance of non-toxigenic diphtheria bacteria for the epidemiology of diphtheria since FREEMAN (1951) was able to prove that a definite relationship exists between toxigenicity and lysogenicity in some strains of *C. diphtheriae*. The work of GROMAN (1955) puts beyond any doubt that with regard to strains C4 and C4 (ß) there can be no question that selection is the predominant factor in the conversion of the non-toxigenic C4 into the toxigenic C4 (ß) strain by means of bacteriophage ß.

Although it was soon established that many diphtheria phages are devoid of the converting property with regard to non-toxigenic strains, the fact that bacteriophages may be able to convert non-toxigenic into toxigenic diphtheria bacteria has led some investigators to think of non-toxigenic bacteria playing a role in epidemiology. According to HEWITT (1952) variations in virulence of infecting organisms might be due to bacteriophages. Entirely new bacterial populations might arise by selection of phage-resistant mutants as well as by conversion. Such populations may be different from the original strains not only as far as virulence is concerned but also with regard to their serological properties. It has been shown that not all phage-resistant mutants are lysogenic. New mutants, possibly with other serological characters, might arise by the action of other phages to which these mutants are susceptible.

The variety of diphtheria strains, differing in serological and cultural properties, may be explained by a selecting role of bacterio-

phages. Two other arguments support this theory, according to Hewitt. First, a variety of strains can often be found in one diphtheria outbreak and even in one patient. Second, different bacteriophages may be obtained from diphtheria strains, isolated during one outbreak. Moreover, Fleck and Kozak (1958) demonstrated the possibility of changing antigenic characters of diphtheria bacteria by lysogenization.

No proof, however, has been given as to a possible role of the conversion phenomenon in epidemiology. In contrast, it has been established (Anderson and Cowles, 1958; Mouton, 1959, 1960) that non-toxigenic diphtheria bacteria can be isolated from cultures of toxigenic, lysogenic strains in vivo as well as in vitro. Pure non-toxigenic strains could be obtained out of toxigenic strains by phage antibodies and inhibition of adsorption of phages to bacteria, which had lost their phages spontaneously in a lysogenic, toxigenic culture, respectively. Because of these results the occurrence of reversion *i.e.* the loss of phages by lysogenic (toxigenic) bacteria in vivo has been suggested to be a possible cause of the frequent isolations of non-toxigenic diphtheria bacteria from convalescents.

In earlier experiments (Mouton, 1959) we attempted to obtain evidence of conversion as a cause of isolated cases of diphtheria by trying to isolate non-toxigenic bacteria from patients in the initial stage of the disease. These experiments, however, were unsuccessful.

Recently we had the opportunity to study a number of toxigenic and non-toxigenic diphtheria strains, which had been isolated from patients in a children's home during an outbreak of diphtheria. This paper deals with some characteristics of these strains. In addition, some tentative explanations will be given for the results of our experiments.

Materials and Methods.

All diphtheria strains mentioned hereafter were isolated in the district laboratory of Public Health at Heerlen[1]) from the throats of patients in a children's home. The strains have been forwarded on Loeffler serum. Before examining the strains, one transfer on Loeffler medium was made.

[1]) We are indebted to Dr. H. Beeuwkes for sending us these strains.

Lysogenicity was determined with the aid of techniques described in an earlier paper (MOUTON, 1959). Both the methods of HEWITT (1952) and THIBAUT and FRÉDERICQ (1952) were applied.

Toxigenicity tests according to ELEK and RÖMER, respectively, were performed, using techniques described in an earlier paper (MOUTON, 1959).

EXAMINATION OF DIPHTHERIA STRAINS.

Strain Nr. 70.

This strain was isolated (30-5-59) from a patient, being the first case in the diphtheria outbreak. The strain proved to be a non-toxigenic mitis type. Out of strain Nr. 76 (toxigenic, gravis), which was isolated from another patient, a phage (76/70) could be obtained by which strain Nr. 70 was lysed. All attempts to demonstrate conversion by this phage failed: strain Nr. 70R76/70 (R = resistant to) proved to be a non-toxigenic mitis type.

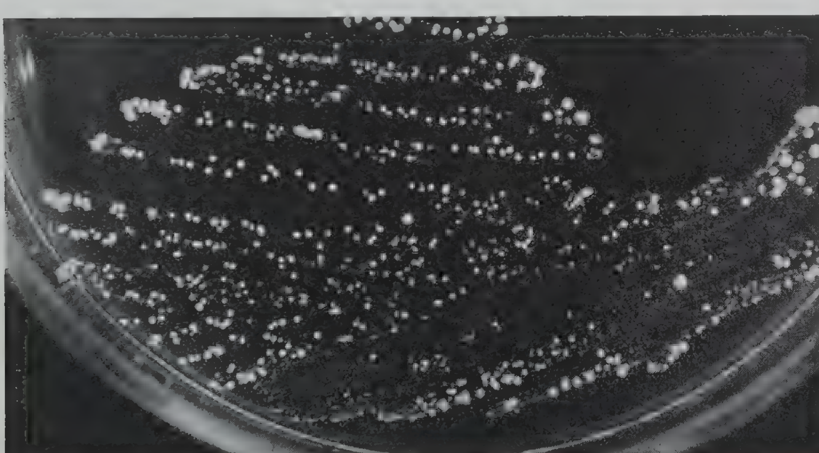

PLATE 1. Culture of strain nr. 70 on H.I.B. agar, characterized by the presence of small colonies with a gelatinous appearance.

After a number of transfers in H.I.B. (Heart Infusion Broth, Difco) strain Nr. 70 lost its susceptibility to phage 76/70. Seeding on H.I.B. agar, performed after some transfers on H.I.B., gave rise to a culture in which many colonies looked like being infected by a phage (Plate I, small colonies). After 24 hours' incubation these colonies, arising among normal ones, were still very small (0.5 mm

diam.) and had a gelatinous appearance. Prolonged incubation (three days) resulted in colonies with a normal aspect. Bacteria of these colonies were not lysed by phage 76/70 and were non-toxigenic, type mitis. Superisolation of the normal colonies and plating hereafter resulted in a culture with the same aspect as the one described above.

Strain Nr. 71.

Strain isolated (30-5-59) from a diphtheria patient. This was a non-toxigenic mitis strain immediately after isolation. Out of strain Nr. 76 (see above) a phage was isolated, which was able to lyse strain Nr. 71. After plating by the Gratia method small turbid plaques were formed. In bacterial growth resistant to this phage non-toxigenic bacteria of the mitis type as well as toxigenic bacteria of the gravis type could be found. The same, however, held true for a culture of this strain, made after several transfers in H.I.B. Plating of a culture on H.I.B. agar before the transfers in H.I.B. resulted in growth, characterized by the presence of small gelatinous colonies, growing out into colonies with a normal aspect in three days. Cultures made of these colonies were not lysed by phage 76/70.

Strain Nr. 72.

Non-toxigenic mitis strain, isolated (1-6-59) from a diphtheria patient. The results of the experiments with this strain were similar to those obtained with strain Nr. 70.

Strain Nr. 73.

Strain isolated (1-6-59) from a diphtheria patient and proved to be a non-toxigenic mitis strain immediately after isolation. After two transfers on Loeffler serum, however, the strain was a toxigenic gravis type. The results of phage susceptibility tests, which followed the same pattern as mentioned for strain Nr. 71, provided circumstantial evidence that contamination or interchanging could be excluded.

Phage 76/73 gave rise to a few turbid small plaques in a culture of strain Nr. 73 (Gratia method). After some transfers in H.I.B. the strain proved to be resistant to this phage.

Strain Nr. 75.

Non-toxigenic mitis strain, isolated (3-6-59) from a nurse who was not suffering from diphtheria. It was not susceptible to a phage,

isolated from strain Nr. 76. Serial transfers in H.I.B. did not influence any properties of the strain.

Strain Nr. 76.

Toxigenic gravis strain, isolated (3-6-59) from a diphtheria patient. Out of a culture of this strain a phage could be obtained, which was able to form plaques in cultures of strains Nrs. 70, 71, 72 and 73, when plated by means of the double agar layer method of Gratia. Isolations of phages active against these strains were performed on each strain separately.

Strains Nrs. 77, 79, 80, 87 and 88.

These are all toxigenic gravis strains, isolated on 8-6 and 11-6-59 from diphtheria patients. Out of cultures of these strains, except Nr. 87, phages could be obtained with lytic activity against strain Nr. 70, before the latter became resistant to phage 76/70 by serial transfers in H.I.B.

Strains Nrs. 81 to 86, 89 to 92 and 94.

These toxigenic gravis strains, also isolated (8-6 and 11-6-59) from cases of diphtheria, occurring during the outbreak of diphtheria in a children's home, were not examined for lysogenicity.

The characteristics of the strains, which were non-toxigenic at isolation, can be found in Table 1.

TABLE 1.

Characteristics of non-toxigenic strains isolated during an outbreak of diphtheria in a children's home, before and after serial transfers in H.I.B.

Strain Nr.		70	71	72	73	75
Toxigenicity test H.I.B. transfers	before	—	—	—	—	—
	after	—	—/+	—	+ [1]	—
Phage susceptibility test H.I.B. transfers	before	+	+	+	+	—
	after	—	—	—	—	—
Type H.I.B. transfers	before	mitis	mitis	mitis	mitis	mitis
	after	mitis	mitis / gravis	mitis	gravis	mitis

[1]) Toxigenic gravis strain after two transfers on Loeffler serum.

Discussion.

When analyzing the results of the experiments, two facts are evident.

First, four out of five strains, originally being non-toxigenic and susceptible to a phage obtained from a culture of strain Nr. 76, lost their susceptibility to this phage by repeated transfers.

Second, there is the observation of gelatinous colonies in an agar culture of three of these strains (Nrs. 70, 71 and 72) after a few broth transfers. These colonies had the same aspect as those obtained by plating a culture of diphtheria bacteria, infected with a temperate phage.

These two facts have led us to the conclusion, that the four strains mentioned above were already contaminated by a phage at isolation. Resistance of the non-toxigenic strains to the isolated phages after a few transfers is in favour of either relationship or identity of the isolated and the "contaminating" phages. In our opinion it is most likely that the "contaminating" phages are identical with the phages obtained from strain Nr. 76.

Two types of diphtheria bacteria (non-toxigenic mitis and toxigenic gravis) were demonstrated simultaneously in strain Nr. 71 only. The results of phage-susceptibility tests and toxigenicity tests of strain Nr. 73, however, make it clear that, besides toxigenic bacteria, non-toxigenic phage-susceptible bacteria have been present in this strain as well.

Conversion appears to be unlikely as a cause of change in virulence of strains Nrs. 71 and 73, because of the very probable identity of the "contaminating" and the isolated phages, the latter of which was shown to be not capable of converting the non-toxigenic strains Nrs. 70 and 72 into toxigenic strains. Isolation of a non-toxigenic phage-resistant strain Nr. 71 cannot be considered to support this view, since non-lysogenic phage-resistant bacteria can originate from a phage-infected culture.

As no converting capacity of the isolated phages could be demonstrated, the occurrence of selection of toxigenic bacteria must be the explanation for the change in virulence of strains Nrs. 71 and 73. A small number of toxigenic bacteria must then have been present in the original cultures of these strains. In that case our methods to determine toxigenicity of diphtheria cultures (modified Elek test and intracutaneous guinea pig test of Römer) have undoubtedly failed with respect to strains Nrs. 71 and 73 at isolation. Yet, it is

not surprising that these tests are not as sensitive as to detect some toxigenic bacteria in a mainly non-toxigenic diphtheria culture.

In view of the facts described above a presumptive cause of events was the following: Bacteria of a non-toxigenic mitis strain, present in a small community, were gradually replaced by bacteria of a toxigenic gravis strain. This process was accelerated *c.q.* accomplished by phages, liberated by lysogenic toxigenic bacteria, with the capacity to lyse the non-toxigenic bacteria, only a small fraction of the latter becoming lysogenic.

Non-toxigenic diphtheria bacteria were isolated out of the very first patients in the diphtheria outbreak. Later on no more of these strains were found. This sequence of isolations during the outbreak can also be explained by selection.

In our opinion it is justifiable to state that the results of our experiments point definitely to an epidemiological significance of the bacteriophage of *C. diphtheriae*, as HEWITT (1952) suggested. Diphtheria phages, indeed, seem to be able to bring about a change in a mixed population of diphtheria bacteria in vivo in a relatively short time.

Questions regarding a possible epidemiological importance of the conversion phenomenon must be left unanswered.

Also the significance of non-toxigenic diphtheria bacteria for epidemiology has not been made clear. Although non-toxigenic diphtheria bacteria can be isolated now and then from healthy individuals, it does not seem probable that the isolation of these bacteria from a few diphtheria patients at the beginning of a local outbreak of diphtheria is coincidental. What is the significance of these bacteria for man? It is still possible that the occurrence of these bacteria in relation to cases of diphtheria has to be explained by conversion and reversion (cf. ANDERSON and COWLES, 1958; MOUTON, 1959) in vivo. The fact that no toxigenic but merely non-toxigenic, phage-resistant bacteria (Nr. 75) were isolated from the one grown-up, probable immune, person in the outbreak may be of significance. Attempts to explain this observation, however, would be based too much on hypothetical grounds. Definite conclusions regarding the place of non-toxigenic diphtheria bacteria in epidemiology of diphtheria will have to be postponed until more data are available.

Summary.

During the beginning of an outbreak of diphtheria in a small community a few non-toxigenic diphtheria strains were isolated from patients.

In two cases non-toxigenic as well as toxigenic diphtheria bacteria were demonstrated in cultures originating from patients.

Experiments with bacteriophages, liberated by toxigenic strains, which were isolated from patients during the same outbreak, as well as the phenomenon that two of the strains, which became phage-resistant after some broth transfers remained non-toxigenic, are not in favour of conversion having taken place in vivo.

Selection of toxigenic bacteria, caused by bacteriophages with lytic activity against non-toxigenic bacteria, is a suggestive explanation for the gradually increasing predominance of toxigenic bacteria during this outbreak.

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THE OCCURRENCE OF A YELLOW, ANTIBIOTIC PIGMENT IN *LASIOSTICTIS FIMBRIATA*

WITH A NOTE ON ITS CONIDIAL STAGE

by

J. GREMMEN

(Received December 14, 1959).

INTRODUCTION.

During continued studies in the inoperculate Discomycetes culture-experiments appeared to be essential in order to determine a connection between ascigerous and conidial stages. For that purpose pure cultures were obtained from ascospores and during development regularly tested as to the occurrence of conidial fructifications. In the course of the work it has been possible to identify a few imperfect stages developed in vitro with similar forms found in nature (GREMMEN, 1960).

This method, merely performed in order to get full information on the life history of these organisms, opened moreover new perspectives on the occurrence of certain metabolites.

In some of these cultures the formation of a crystalline substance has been observed. These crystals are generally hyaline-coloured and often found between the growing hyphae (sometimes stored in considerable quantities in fructifications); seldom yellowish or yellow-orange. Special strains have been found to be productive, whereas others lack this property.

Particular interest has been given to these yellow-orange pigments, because of their antagonistic properties. The present paper describes a new case of a yellowish metabolite formed by a fungus, which in the writer's opinion will be found to occur more frequently as soon as it is consciously looked for. Up to the present time reports on such pigments are very scanty and all available information will be presented.

A first report concerning a yellow-coloured pigment in a discomycetous fungus dates from Wood (1953). He investigated the fungus *Lambertella corni-maris* Höhnel which was isolated from the fruits of apple and pear. This fungus inhibits the growth of *Botrytis cinerea* on agar, showing the greatest antagonism in experiments on glucose-peptone agar with pH 4.0-5.0. On certain media, particularly on potato-glucose agar, clusters of orange-yellow crystals were formed which were not chemically investigated. Besides antagonism against *Botrytis cinerea* this substance demonstrates a striking growth inhibition for some other fungi and bacteria as *Staphylococcus aureus*, *Mycobacterium phlei* and *Bacterium coli*.

Cultures of *Lambertella corni-maris* (Nr. 189, a, b) have been received formerly by the present author. They have been under observation in the laboratory for many years and are still in excellent condition. The pigment formation, however, is still very meagre.

In 1956 Gremmen reported on a crystalline, antibiotic substance observed in cultures of the fungi *Mollisia caesia* Sacc. *sensu* Sydow (Nr. 69 a, 170) and *Mollisia fallens* (Karst.) Karst. (Nr. 71b). These fungi were isolated from last year's branches of *Salix* and *Tilia*, all producing lemon- to sulphur-coloured masses of crystals showing pronounced inhibition against a number of fungi such as *Fomes annosus*, *Dothichiza populea*, *Sclerotinia trifoliorum*, *Sclerotinia minor*, *Pollaccia radiosa*, *Cladosporium* and *Penicillium* species. Strain Nr. 170 with the highest pigment production was investigated in the Institute for Organic Chemistry T.N.O. at Utrecht. It was demonstrated that the yellow substance is a dichloronaphthoquinone derivative, not previously encountered and designated mollisin (van der Kerk and Overeem, 1957).

Recently a description of a characteristic pigment in a Discomycete came under the writer's notice (Read, Shu, Vining and Haskins, 1959). This paper deals with a bright orange-red pigment obtained in a culture of a hitherto unknown species isolated from a diseased pine-seedling. This substance inhibits Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, but does not show activity against Gram-negative organisms such as *Escherichia coli*. The compound showed quinonoid properties forming a monoquinoxaline derivative and was designated mycochrysone.

Read *et al.* refer to a paper by Anderson and Murray (1956) suggesting a close relationship between mycochrysone and 4 : 9-dihydroxyperylene-3 : 10-quinone isolated from the Ascomycete

Fig. 1. Culture of *Verticillium albo-atrum* (left) combined with *Lasiostictis fimbriata*, strain 342. Antagonistic action is very characteristic. Black background. On malt-dextrose-peptone agar. (*Foto Bosbouwproefstation*)

Daldinia concentrica (Bolt) Ces. & de Not. This substance was obtained by extraction of the fruit-bodies; not from cultures. Further research concerning antibiotic activity of this compound was not performed.

Again very recently the present author came across a discomycetous fungus forming yellow and yellow-orange crystals in a pure culture. This organism was collected in the course of a study bearing on pinicolous Ascomycetous fungi and proved to be identical with *Lasiostictis fimbriata* (Schw.) Bäumler. Isolations of the fungus (Nr. 342) [1]) were obtained from ascospores of apothecia inhabiting old cones of *Pinus sylvestris*. The pigment demonstrates affinities with mollisin since the crystals readily dissolve in aqueous

[1]) Number of culture Bosbouwproefstation.

NaOH forming a purplish red solution. Subcultures of this strain were handed over to Prof. Dr. G. J. M. VAN DER KERK (Institute for Organic Chemistry T.N.O., Utrecht) for identification of the compound.

ANTAGONISTIC PROPERTIES.

Antifungal activity of the yellow pigment has been observed in vitro. This has been tested in the same way as described in an earlier paper (GREMMEN, 1956).

Antagonistic action was demonstrated against some fungi, such as *Ophiostoma canum* (Münch) H. & P. Sydow; *Verticillium albo-atrum* Reinke & Berth. (fig. 1); *Pollaccia radiosa* (Lib.) Bald. & Cif. and a *Penicillium* species.

THE ORGANISM.

The organism is a member of the Ascomycetes and must be classified among the inoperculate Discomycetes. Its correct name is: *Lasiostictis fimbriata* (Schw.) Bäumler, in Beitr. Crypt. flor. Preszburg **3**, 39, 1897.

syn. *Stictis fimbriata* Schw., in Syn. fung. Am. bor. 1832.
syn. *Lasiostictis conigena* Sacc. & Berl., in Miscell. myc. **2**, 24, 1885.
Imperfect stage: *Eriosporopsis albida* Petrak, in Sydowia **1**, 94, 1947.

Description:

Apothecia 0.3-0.8 mm diameter (fig. 2,I), primarily initiating in the host, at maturity rupturing the host tissue in a crateriform manner, with a dark-brown disk and surrounded by a white fringe. Hymenium and hypothecium colourless. Exciple consisting of a dark-brown stratum tectricum provided with colourless interascicular hyphae; a basal part of this tissue can be hardly distinguished. Asci (95-125) $\times$ (10-11)μ, papillate, surrounded by colourless, filiform, paraphyses, width about 2 μ. Ascospores (77-88) $\times$ (2.5-3) μ, colourless, 1- or more-celled, generally curved or vermicular, rarely straight and intertwined in the ascus.

Hab. On old cones of Scots pine (*Pinus sylvestris*), lying on the ground-floor, 25 VI 1959, near Bristen, Maderanertal, Switzerland (GREMMEN, 1656) [1]).

A few months afterwards apothecia of *Lasiostictis fimbriata* were collected for the first time in the Netherlands. They were found growing on old cones of austrian pine (*Pinus nigra* var. *austriaca*), 28 X 1959, in the dunes near Noordwijk aan Zee (GREMMEN, 1685)[1]).

[1]) Herbarium-number.

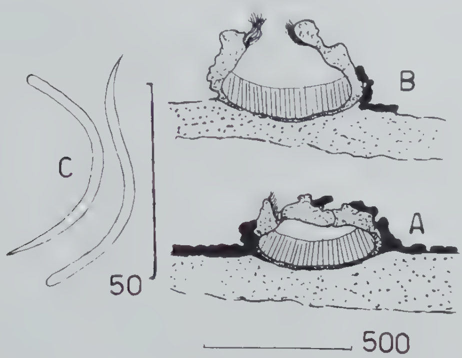

Fig. 2. I, *Lasiostictis fimbriata* (Schw.) Bäumler. A-B, Apothecia; C, Ascospores.

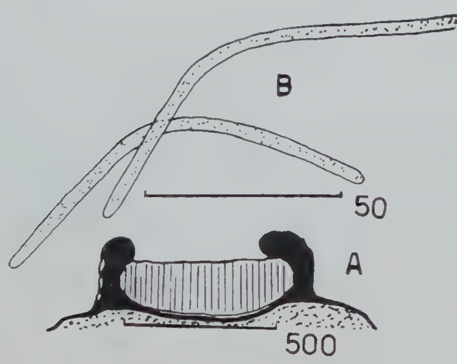

Fig. 2. II, *Naemacyclus niveus* (Pers.) Sacc. A, Apothecia; B, Ascospores.

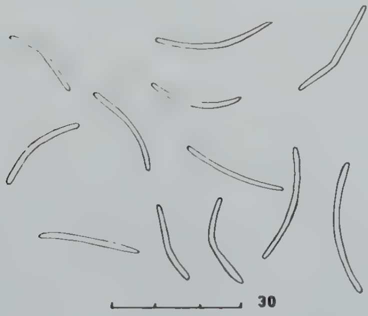

Fig. 2. III, *Lasiostictis fimbriata* (Schw.) Bäumler. Conidial stage (*Eriosporopsis albida* Petrak).

Life history.

Petrak (1947a) in a detailed study on the fungi *Naemacyclus niveus* (Pers.) Sacc. and *Lasiostictis fimbriata* (Schw.) Bäumler discussed their structural similarity.

Since he found both fungi on pine — even on the same trees — he wondered whether they are specifically distinct or merely substrate-forms. He considers *Lasiostictis* Sacc. & Berl. as identical with *Naemacyclus* Fuck.

Culture-experiments performed by the present author with these fungi indicate that actually they are different species, *Naemacyclus niveus* only occurring on pine-needles, whereas *Lasiostictis fimbriata* lives on the old cones.

In the same year Petrak described a pycnidial fungus named *Eriosporopsis albida* Petr., which was collected by him on cones of Scots pine. It appeared that these conidial fructifications were now and then associated with the apothecia of *Lasiostictis fimbriata* and so Petrak supposed the first fungus to be the imperfect form of the latter (Petrak, 1947b). By means of culture-experiments Petrak's assumption has been confirmed.

Culture-Studies.

Both organisms, *Lasiostictis fimbriata* and *Naemacyclus niveus*, have been studied in pure culture. They show characteristic differences in vitro.

a) Nr. 342, Swiss strain of *L. fimbriata*:
After rapid germination of the ascospores initial hyphal growth was slow, increasing later on, forming a pure white, slightly floccose mycelium on plain maltagar. Afterwards black pustules were observed in the medium, but although they did not mature they certainly represent the conidial stage. The perfect form was not obtained in culture.

b) Nr. 354, Dutch strain of *L. fimbriata*:
The ascospores germinated quickly, but mycelium growth was extremely slow, forming minute white or grey-coloured colonies on plain maltagar. In the centre of these colonies black pustules grown over by aerial hyphae were observed afterwards. At maturity these pycnidia ooze milky-coloured spore-horns consisting of conidia being (19-30) $\times$ 1 μ (mean average (23-26) $\times$ 1 μ). They are colourless, 1-celled and generally curved or falcate with rounded ends

Fig. 3. *Culture of Verticillium albo-atrum* (left) combined with *Lasiostictis fimbriata*, strain 342. There is no sign of antagonistic action. Black background. On plain maltagar. (*Foto Bosbouwproefstation*)

(fig. 2, III). This stage proved to be identical with *Eriosporopsis albida* Petr. The perfect form was not obtained in vitro.

c) Nr. 269, British strain; Nr. 278, Dutch strain of *N. niveus*: This fungus forms a moderately growing white, afterwards slightly pink-or cream-coloured mycelium with characteristic yellow or yellow-brown patches on plain maltagar. Later on a great number of mature, yellow-green apothecia develop (fig. 2, II), but a conidial stage has never been observed in the cultures.

When small blocks of plain maltagar with mycelium from isolate Nr. 342 were transferred to the same medium enriched with a carbon source (dextrose) yellow crystals were formed around the colony. When moreover peptone was added the amount of pigment still increased and dense clusters of crystals were formed under-

neath as well as around the colony. This stimulation of pigment production was however attended with a marked reduction in mycelium growth of the fungus, whereas a pronounced inhibition against other fungi was observed.

Although the number of strains of *Lasiostictis fimbriata* under observation has been very small up till now, even these two strains are of remarkably distinct behaviour.

When growing isolate 342 without dextrose the yellow metabolite (fig. 3) was never formed, although a great many of hyaline crystals were noticed between the growing hyphae. After adding dextrose (with or without peptone) the yellow substance was abundantly formed (fig. 1).

When cultivating isolate 354 on plain maltagar hyaline crystals were observed too, but after transferring this mycelium to the same medium with dextrose (with or without peptone) the yellow pigment did not occur. Even on maltagar with higher concentrations of dextrose and peptone the result with strain 354 was negative.

Although these few observations may contribute to our knowledge on the existence of certain metabolites with antagonistic properties in fungi, they demand for intensive further research in this field.

Consequently the investigation of a great many fungi of different groups, particularly Ascomycetes (*i.c.* Discomycetes) is necessary. Since physiological differences have been found in morphologically identical species this work requires the investigation of as many strains as possible of the same taxon in order to trace such substances.

Summary.

A second report on a yellow, crystalline, antibiotic substance obtained from a fungus has been given. This pigment was observed in pure cultures of the Ascomycete *Lasiostictis fimbriata* (Schw.) Bäumler. Two strains of this organism have been studied: A Swiss strain (Nr. 342) abundantly producing the substance in vitro and a Dutch strain (Nr. 354) failing this property. It was observed that strain Nr. 342 was not able to form this compound on plain maltagar, but the addition of dextrose was essential. A mycological description of the organism and its conidial stage, *Eriosporopsis albida* Petr. has been published. *Lasiostictis fimbriata* (Schw.) Bäumler has

been compared with *Naemacyclus niveus* (Pers.) Sacc. They have been proved non identical.

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TORULOPSIS DOMERCQII NOV. SPEC.

by

J. P. VAN DER WALT and **AMELIA E. VAN KERKEN**

(Received March 28, 1960).

During studies of the yeast flora associated with cellar equipment in the course of investigations of yeast hazes in finished wines, a new yeast species was isolated from a vat. A very characteristic feature of this species is the smallness of its cells. The following description of the species is based on the standard methods of LODDER and KREGER-VAN RIJ (1952) and WICKERHAM (1951).

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are round, short oval to long oval (1.5 — 3.0) × (2 — 4) μ, single, in pairs, chains or small clusters. After 1 month at 17°C., a sediment and ring are formed.

Growth on malt agar: After 3 days at 25°C., the cells have the same shape and size as in malt extract. After 1 month at 17°C., the streak culture is cream to light brown, butyrous, shiny, flat with delicate structure in the centre of the streak. The margin is smooth.

Slide cultures: No pseudomycelium is formed.

Fermentation: Absent.

Carbon assimilation: Glucose, L-sorbose, D-ribose, ethanol, glycerol, D-mannitol, D-sorbitol, potassium D-gluconate, succinic acid, malic acid and citric acid are assimilated. Galactose, maltose, saccharose, cellobiose, trehalose, lactose, melibiose, raffinose, melizitose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, erythritol, adonitol, dulcitol, α-methyl-D-glucoside, salicin, DL-lactic acid and m-inositol are not assimilated.

A s s i m i l a t i o n o f n i t r a t e a n d n i t r i t e: Positive.
S p l i t t i n g o f a r b u t i n: Absent.
G r o w t h i n t h e a b s e n c e o f a n e x t r a n e o u s v i t a m i n s o u r c e: Absent.

Discussion.

In view of the absence of ascospore and pseudomycelium formation, the species is assigned to the genus *Torulopsis* (*in sensu* Lodder et Kreger-van Rij). Several nitrate-positive species have been included in this genus, but none of these corresponds with that described above. Within this genus the new species is perhaps most closely allied to *Tor. magnoliae* Lodder et Kreger-van Rij.

Since Wickerham (1958) had shown that the nitrate-positive species *Torulopsis globosa* (Olsen et Hammer) Lodder et Kreger-van Rij is in fact merely the haplophase of the heterothallic species *Citeromyces matritensis* Santa Maria (Santa Maria, 1957) it seems advisable that all the nitrate-positive *Torulopsis* species be examined for heterothallism. Unfortunately, only one strain of the above described species has as yet been isolated.

For this new species the name *Torulopsis domercqii* is proposed in honour of Dr. Simone Domercq in recognition of her extensive studies on the wine yeasts of Gironde.

A culture of *Torulopsis domercqii* has been deposited in the yeast collection of the Centraal Bureau voor Schimmelcultures in Delft.

Torulopsis domercqii nov. spec.

In extracto malti cellulae rotundae, ovoidiae, longovoidiaeque (1.5-3.0) × (2.0-4.0) μ, singulae, binae et plerumque in catenatae aut in racemis parvis. Sedimentum et anulus formantur.

In agar malti cellulae formae et dimensiones cellularum eaedem sunt quae in extracto malti. Cultura (post unum mensem, 17°C.) flavifusca, mollis, nitida, plana cum structura subtili in parte media. Margine glabro.

Pseudomycelium nullum.

Fermentatio nulla.

Glucosum, L-sorbosum, D-ribosum, ethanolum, glycerolum, D-mannitolum, D-sorbitolum, kalium D-gluconicum, acidum succinicum, acidum malicum et acidum citricum assimilantur at non galactosum, maltosum, saccharosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinosum, melizitosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, erythritolum, adonitolum, dulcitolum, α-methyl-D-glucosidum, salicinum, DL acidum lacticum nec m-inositolum.

Kalium nitricum et natrium nitrosum assimilantur.
Arbutinum non finditur.
Necessariae ad crescentiam sunt vitaminae externae.
Isolata ex cupa vini.

Acknowledgement.

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(National Institute of Public Health, Utrecht, Netherlands).

A SIMPLIFIED METHOD FOR THE PREPARATION OF REITER PROTEIN ANTIGEN

by

J. H. DE BRUIJN

(Received February 12, 1960).

INTRODUCTION.

Since the appearance of our paper dealing with the application of the Reiter protein antigen in the serodiagnosis of syphilis (DE BRUIJN, 1957), complement fixation tests employing this antigen have been subject to a great number of serological evaluations. In most instances, the antigen was prepared according to the directions given by D'ALESSANDRO and DARDANONI (1953), *i.e.* lysis of the treponemes by a number of freezing-thawing cycles and salting-out of the antigen by dialysis against ammonium sulfate solutions of increasing concentrations (DE BRUIJN, 1957; CANNEFAX and GARSON, 1957; WALLACE and HARRIS, 1958). The present paper gives some simplifications in the preparation of this antigen.

MATERIALS AND METHODS.

Cultivation of the treponemes.

The Reiter strain of *Treponema pallidum* is cultured and a suspension of the organisms prepared according to the method of D'ALESSANDRO and DARDANONI (1953) as previously described (DE BRUIJN, 1957).

Desintegration of the treponemes.

The time-consuming cryolysis was substituted by ultra-sonic desintegration (10 KHz, 250 W) during 10 minutes. After centrifugation of the lysate, the latter method left much less solid material as the former one. This fact has not only to be explained by a greater efficiency of the ultra-sonic desintegration but also by a partial

dissociation of the antigenic complex on freezing as observed by PILLOT and FAURE (1959).

Isolation of the protein antigen.

The stepwise salting-out procedure was substituted by a method in which 60 ml of saturated ammonium sulfate solution, previously adjusted to pH 7.4 with ammonium hydroxide, is added to 20 ml ultra-sonic lysate under continuous stirring. After refrigeration during 1 hour, the mixture is centrifuged, the supernatant discarded and the precipitate dissolved in 10 ml 0.1 M glycine-saline buffer (pH 7.4) [1]. The slightly turbid solution is dialysed against several portions of fresh solvent until ammonium sulfate is no longer detectable. Finally, the dialysed solution is centrifuged at 4000 g during 30 minutes, the sediment discarded and the supernatant retained as the antigen.

Chemical characterization of the protein antigen.

Recently, the Reiter protein antigen was shown to be a lipopolysaccharide-protein complex of which only the protein component is responsible for the reaction with syphilitic serum (DE BRUIJN, 1959). For the quantitative estimation of this protein component, the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) was chosen. The color intensity with Folin-Ciocalteau reagent obtained with protein antigen is compared spectrophotometrically with that obtained with a standard tyrosine solution and the result expressed as tyrosine-N.

Freeze-drying of the protein antigen.

Protein antigen, prepared by salting-out of an ultra-sonic lysate of treponemes and dissolving in veronal-buffered saline (DE BRUIJN, 1958), appeared to become insoluble after freeze-drying. In the present method, instead of veronal-buffered saline 0.1 M glycine-saline buffer is used because of its hydrophilic effect (WUNDERLY, 1954), which could be enhanced by the addition of 0.5% gelatine prior to freeze-drying. Besides its promoting effect on the solubility, gelatine proved to be a stabilizing agent for diluted protein antigen. Gelatine has been used before as a preservative for sheep erythro-

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[1]) Glycine 7.5 g, sodium chloride 5.8 g, sodium hydroxide solution (0.1 N) 5 ml, and distilled water to make 1 l.

cytes in the Kolmer test by STEIN (1952). Until now, no 'biologically false positive' results, due to a reaction between gelatine and human serum, have been observed.

Reconstitution of lyophilized protein antigen is promoted by heating in a 37° C. waterbath for some minutes.

Complement fixation test.

The CFT used is a highly standardized routine technique (DE BRUIJN, 1958). The serum was a lyophilized human syphilitic serum. Appropriate serum- and antigen controls were included in the test.

RESULTS AND DISCUSSION.

Protein antigen, prepared according to the new method, was tested in parallel on syphilitic serum with protein antigen, prepared as previously described (DE BRUIJN, 1957). From the result (table 1) it can be concluded that the level of reactivity is the same in both cases. The new preparation seems to be more concentrated, which is in accordance with the result of the chemical analysis. The parallelism observed between serological activity and N-content as determined colorimetrically with the Folin-Ciocalteau reagent may be the first step on the way of chemical standardization, which is so urgently needed in the case of Reiter protein antigen.

TABLE 1.

Parallel titration on syphilitic serum of two Reiter protein preparations.

Serum dilutions	Antigen (new) dilutions					Antigen (old) dilutions				
	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
1 : 10	++++	++++	++++	++++	+++	++++	++++	++++	++++	+
1 : 20	++++	++++	++++	++++	++	++++	++++	++++	+++	—
1 : 40	++++	++++	++++	++++	+	++++	++++	++++	++	—
1 : 80	—	++	+++	+	—	+	+++	+++	—	—
1 : 160	—	—	—	—	—	—	—	—	—	—
	Tyrosine-N content of undiluted antigen: 59 γ/ml					Tyrosine-N content of undiluted antigen: 45 γ/ml				

Summary.

A description is presented of a simplified method for the isolation of the protein fraction from *Treponema pallidum* (Reiter strain), *i.e.* ultra-sonic desintegration of the treponemes and salting-out of the

protein antigen by adding ammonium sulfate until 75% saturation. To promote redissolving after freeze-drying, glycine-saline buffer and gelatine are used. A parallelism between serological activity and N-content as determined colorimetrically with the Folin-Ciocalteau reagent is demonstrated.

Acknowledgement.

The author is indebted to Mr. S. de Bruijn and Miss J. H. L. de Jong for skilful help.

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THE ROLE OF COBALT IN AMINO ACID ANTAGONISMS [1])

by

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(Received April 1, 1960).

INTRODUCTION.

Antimicrobial activities of numerous metal binding organic compounds often can be enhanced by specific metallic ions (ALBERT, 1958; WEINBERG, 1960). For example, L-cysteine, 8-hydroxyquinoline, isoniazid, sodium dimethyldithiocarbamate, and streptomycin can be enhanced by copper; ethylene diamine tetraacetic acid and penicillin by cobalt; aspergillic acid, L-cysteine, and 8-hydroxyquinoline by iron; the tetracyclines by manganese and magnesium; and bacitracin by zinc. Conversely, the activities of these compounds can sometimes be suppressed by different concentrations of the same metallic ions, by certain other metallic ions, or by various other metal binding agents (ALBERT, 1958; WEINBERG, 1960).

Inasmuch as amino acids are good metal binding agents, it is possible that the enhancement of the bacteriostatic activity of one amino acid by specific metallic ions could be prevented by the binding of the ions by a second amino acid. Such a mechanism might be involved in some cases of an antagonism of one amino acid towards another. To determine if situations of this type could actually exist, the following study was undertaken.

MATERIALS AND METHODS.

Bacterial Strains and Culture Media. The test strains of bacteria consisted of a stock culture strain each of *Sta-*

1) Supported by a research grant (E-2252) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

phylococcus aureus (coagulase positive) and *Escherichia coli*. The culture medium consisted of nutrient agar which was prepared by dissolving 0.5% polypeptone (Baltimore Biological Laboratories), 0.3% beef extract (Difco), and 2.0% flake agar (Baltimore Biological Laboratories) in distilled water. The medium was sterilized by exposure to 121° C. for 15 minutes.

Amino Acids and Metallic Salts. The amino acids employed in this study consisted of DL-alanine, β-alanine, L-arginine HCl, L-aspartic acid, L-cysteine HCl, glycine, L-histidine, DL-homoserine, DL-isoleucine, L-leucine, L-lysine, DL-methionine, L-proline, D-serine, L-serine, DL-serine, D-threonine, L-threonine, DL-threonine, L-tryptophane, L-tyrosine, and L-valine. With the exception of DL-homoserine which was obtained from Nutritional Biochemicals Corporation, each chemical was procured from California Corporation for Biochemical Research. The metallic salts consisted of analytical grades of $Co(NO_3)_2.6H_2O$ (Baker), $Cu(NO_3)_2.3H_2O$ (Mallinckrodt), $CuSO_4$ (Merck), and $CuCl_2.2H_2O$ (Mallinckrodt). Each solution was sterilized by filtration and adjusted to pH 7.0 prior to use.

Experimental Procedures. To detect enhancement of the bacteriostatic action of an amino acid by salts of cobalt or copper, the double gradient plate method (Weinberg, 1957) was employed. In this method, the metallic salt was included in the first or lower layer of the agar medium (solidified while the plate is in an inclined position) and the amino acid was incorporated in the second or upper layer (solidified while the plate is in a level position). Single gradient plates containing either the metallic salt or the amino acid were routinely included as controls. The maximum, non-toxic concentration of each test substance for each bacterial strain was determined in preliminary trials with single-gradient plates. These and lower concentrations were then used in the double gradient plate experiments.

To determine the effect of a second amino acid on the combined bacteriostatic action of the first amino acid and a metallic salt, conventional single level layer plates were employed. Varying combinations of the first amino acid and the metallic salt were incorporated in the agar, and the second amino acid was contained in a filter paper disk placed on the surface of the agar immediately

following inoculation. In other experiments, one of the two amino acids was incorporated in the agar and the second amino acid placed on a filter paper disk; an adjacent disk contained the metallic salt.

The inocula consisted of approximately 1×10^3 viable cells obtained from 24 hour broth cultures. The cells of the inocula were spread evenly over the solidified agar surfaces. The inoculated cultures were incubated at 37° C. for at least 48 hours. Observations of the extent of growth were made after 24 and 48 hours and, in some cases, after 72 hours had elapsed.

Experimental results.

The results obtained with the double gradient plates indicate that with *Staphylococcus aureus*, the antibacterial action of L-cysteine was strongly enhanced by copper and the antibacterial action of L-serine and to a lesser extent of D-serine was moderately enhanced by cobalt. Solutions of the nitrate, sulfate, and chloride of copper were equally active. In the absence of the addition of the metallic salts to the medium, a twenty-five fold increase in concentration of L-cysteine and a ten fold increase in concentration of L-serine was required to achieve the same bacteriostatic effect that was obtained with combinations of the respective amino acids and metallic ions. Results obtained with double gradient plates containing selected concentrations of L-serine and cobalt are presented in Fig. 1. Molar ratios of L-cysteine to copper of between 10 and 100 to 1 and of L- or D-serine to cobalt of between 25 and 250 to 1 possessed maximum bacteriostatic activity. No enhancement by the metal ions of the toxicity of any of the other amino acids was obtained. With *Escherichia coli*, the metallic salts plus L-cysteine and L- or D-serine as well as each of the other amino acids were completely inactive. The bacteriostatic activity against *S. aureus* demonstrated with double gradient plates, as well as with single level layer plates described below, was clearly evident at 24 hours, was partially diminished at 48 hours, and had completely disappeared at 72 hours.

Inasmuch as the mutual effects of sulfur containing amino acids and copper have been studied previously (Woiwood, 1954), it was decided to concentrate during the remainder of the investigation on the synergistic anti-staphylococcal action of L- or D-serine and cobalt. Since of the natural amino acids, L-histidine and L-cysteine

Figs. 1, 2 and 3.

In each of the three figures, the shaded areas represent visible growth of *Staphylococcus aureus* after 24 hours at 37° C. The quantities listed of L-serine or cobalt added to the medium are the final concentrations of the added material in the nutrient agar. The quantities listed for cobalt are the concentrations of cobaltous ion rather than cobaltous chloride.

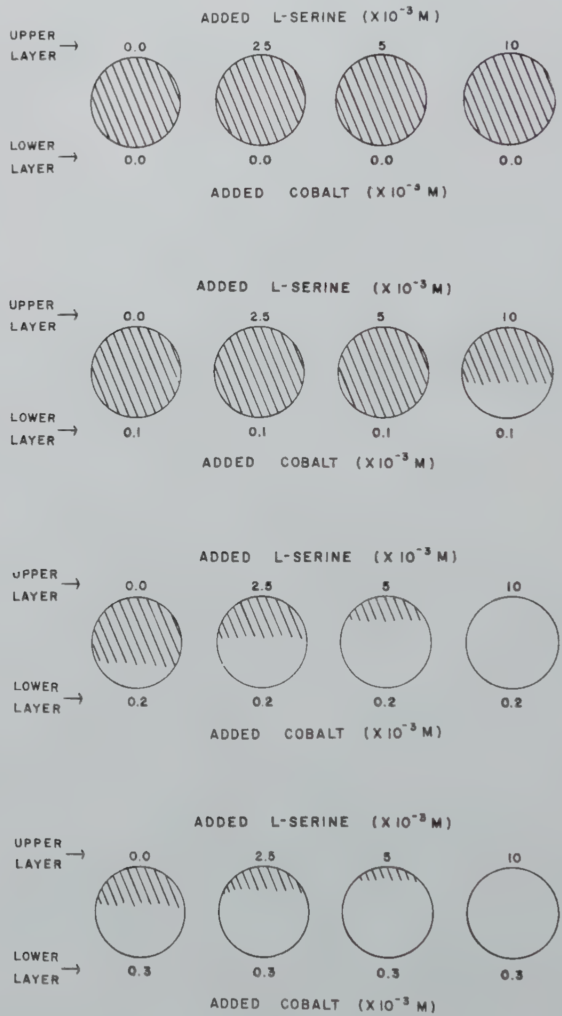

Fig. 1.

Mutual enhancement of the bacteriostatic action of L-serine and cobalt demonstrated with double gradient plates. The metallic salt is incorporated in the first or lower layer and the amino acid in the second or upper layer.

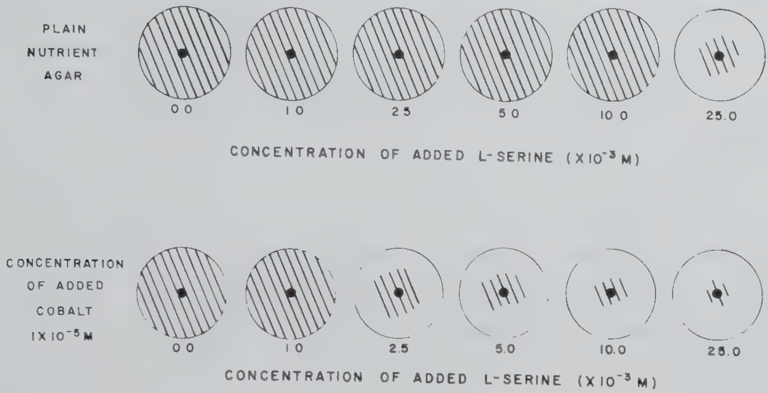

Fig. 2.

Reversal of the bacteriostatic action of L-serine and cobalt by L-histidine demonstrated with single level layer plates. The nutrient agar in the top row of plates contains added L-serine, no cobalt, and disks impregnated with L-histidine. The nutrient agar in the bottom row of plates contains added L-serine and cobalt plus disks impregnated with L-histidine. Each disk contains 0.05 ml of a 0.25 M solution of L-histidine.

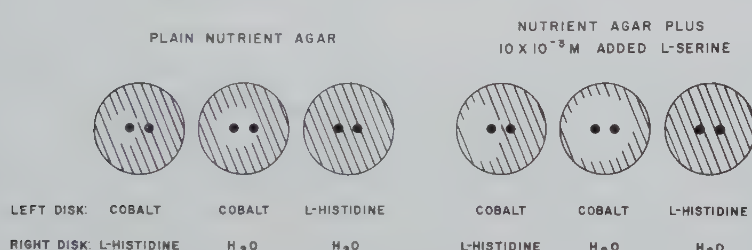

Fig. 3.

Reversal of the bacteriostatic action of L-serine and cobalt by L-histidine demonstrated with single level layer plates. The nutrient agar in three of the six plates contains added L-serine. The disks have been impregnated with either cobalt, L-histidine, or distilled water. Each disk of cobalt contains 0.05 ml of a 0.02 M solution. Each disk of L-histidine contains 0.05 ml of a 0.25 M solution.

are the most efficient suppressors of cobalt toxicity (Schade, 1949), experiments were performed with single level layer plates in which cells of *S. aureus* were exposed to varying combinations of L- or D-serine, cobalt, and L-histidine or L-cysteine. Results of some of these experiments are presented in Figs. 2 and 3. It may be observed that L-histidine was quite active in suppressing the bacteriostatic

action of L-serine plus cobalt. Comparable results were obtained with L-histidine, cobalt, and D-serine and with L-cysteine, cobalt and L- or D-serine. In every experiment, however, the cobalt enhancing effect on D-serine was considerably less than on L-serine. Subsequent tests with each of the other amino acids demonstrated that none could replace L-histidine or L-cysteine in this system. In the absence of added cobalt, L-histidine and L-cysteine had a distinct but much less pronounced antagonistic action toward L- or D-serine.

Discussion.

Chenoweth (1956) has observed that "a particularly high activity of histidine or cysteine among the amino acids found to influence a given phenomenon suggests that sequestration by chelation is at the root of the phenomenon". Likewise, metal binding is indicated as the cause or as a contributing factor if a given effect can be produced by several chemically distinct metal complexing agents. And finally, a non-enzymatic reaction such as metal binding should be considered as a factor in the production of an effect in which both the L- and D-isomers of an amino acid are active. Thus, the comparable bacteriostatic action of thioglycollic acid, L-serine, D-serine, L-cysteine, and glycine towards *Staphylococcus aureus* observed by Castellani (1953) might well depend on an appropriate concentration of a metallic ion such as cobalt or copper in the various cream pastry fillings examined. Castellani (1953, 1955) noted that the activity of serine was unaffected by pineapple, significantly affected by milk and egg, and completely suppressed by coconut and cocoa. It would be of considerable interest to ascertain the variation in concentration of cobalt and of such cobalt-binding agents as histidine in these food substances.

With regard to the present study, it is known that the polypeptone in nutrient agar contributes approximately 3.0×10^{-6} M cobalt and copper, respectively, to the medium; unfortunately, data are not available concerning the concentration of these metallic ions in either the meat extract or agar components of the medium. The moderate antagonistic effect of L-histidine and L-cysteine on serine toxicity in this medium may result from the ability of the two former amino acids to compete successfully with the latter for cobalt in the nutrient agar. Thus when additional cobalt is placed in the

medium, both the toxicity of serine and the antagonistic effect of cysteine and histidine are considerably intensified.

WOIWOOD (1954) has noted that the autoclaving of a mixture of cysteine and copper resulted in the formation of sufficient copper sulfide to inhibit growth of *S. aureus* but not of *E. coli*. In contrast, a mixture of autoclaved copper and non-heated cysteine (in peptone) was non-toxic. In the present study, however, copper strongly enhanced the bacteriostatic action of filter-sterilized solutions of L-cysteine toward *S. aureus* and it is believed that, under the conditions of the present experiments, the actual toxic entity is the copper complex of the amino acid rather than copper sulfide.

In the experiments of CASTELLANI (1953) and WOIWOOD (1954), as well as in the present studies, gram negative bacteria were not inhibited by either the active amino acids or amino acids plus metallic ions. This situation is, of course, similar to inhibition by synergistic combinations of 8-hydroxyquinoline and iron, of bacitracin and zinc, and of penicillin and cobalt. With each of the drug-metal complexes, gram negative bacteria are considerably more resistant than gram positive organisms.

The toxic action of serine plus cobalt or cysteine plus copper would appear to be different from that of metal complexes of either 8-hydroxyquinoline, bacitracin or penicillin since, in unpublished experiments, we have observed that as with the two antibiotics, cell wall synthesis is suppressed by 8-hydroxyquinoline but not by L-serine or L-serine plus cobalt. As with 8-hydroxyquinoline, however, the amino acids may act merely by carrying into the sensitive cells quantities of toxic metallic ions that cannot be tolerated by sensitive enzymes; alternately, the metallic ions may serve as a bridge to unite either 8-hydroxyquinoline or toxic amino acids with enzyme surfaces. In either case, impairment of enzymatic activity is sufficient to cause a significantly prolonged lag phase of growth.

Summary.

The bacteriostatic action towards *Staphylococcus aureus* of L-cysteine is enhanced strongly by copper and of L- and D-serine by cobalt. The serine-enhancing activity of cobalt is completely suppressed by either L-histidine or L-cysteine. It is suggested that metal binding may play a role in some instances of amino acid

antagonism, especially when one member of the pair is either histidine or cysteine.

Acknowledgement.

It is a pleasure to acknowledge the capable technical assistance of Misses Ann L. Bockstahler, Judith I. Brooks, Judith K. Donaldson, and Joyce A. Lockhart.

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(From the National Institute of Public Health, Utrecht, The Netherlands).

TWO NEW *SALMONELLA* TYPES (*S. ROTTERDAM* AND *S. BLIJDORP*)

by

P. A. M. GUINÉE and E. H. KAMPELMACHER

(Received March 30, 1960).

In January 1959 *Salmonella rotterdam* was isolated from the feces of a tree-agame (*Calotes versicolor*) by P. ZWART (Institute for Tropical and Protozoan Diseases, State University, Utrecht). This animal belonged to the Rotterdam Zoo. *S. blijdorp* was isolated at the same time from the organs of a cameleon (*Chameleon jacksoni*), also by P. ZWART in the above mentioned Zoo.

Both cultures possessed the morphological and biochemical characteristics of the genus *Salmonella*. The organisms were motile and Gram-negative and did not grow in Møller's KCN-medium. The biochemical behaviour was as follows: no fermentation of lactose, salicin, sucrose and adonitol. No production of indol and no hydrolysis of urea. Rapid fermentation of inositol, dulcitol, glucose (with gas) and mannitol. *S. rotterdam* liquefied gelatin within three days; *S. blijdorp* gave no liquefaction of gelatin. There was rapid fermentation of maltose by *S. rotterdam*, whereas *S. blijdorp* fermented maltose late and irregularly. Both cultures produced H_2S, the Voges-Proskauer reaction was negative and the methyl-red reaction positive.

Examined serologically, *S. rotterdam* was agglutinated to titer (1 : 1280) by *S. poona* O serum and in absorption tests removed all agglutinins of that serum to the titer 1 : 20. O serum *S. rotterdam*, absorbed with *S. poona*, gave no agglutination with *S. poona* antigen, whereas *S. rotterdam* still agglutinated to 1 : 20. Therefore the culture belonged to O group F of the genus *Salmonella*; it contained the somatic antigens 1, 13, 22.

The H-antigens were diphasic. Phase 1 was agglutinated to titer (1 : 10.000) by the H-antigen of *S. budapest* (g, t). In absorption

tests the agglutinins were removed to 1 : 80. Phase 2 was agglutinated to the titer of (1 : 5000) and in adsorption tests removed all agglutinins from serum, derived from phase 2 of *S. cholerae suis* (1,5).

The antigenic formula of the type examined was therefore 1, 13, 22 : g, t : 1,5.

Examined serologically S. *blijdorp* was agglutinated to titer (1 : 640) by *S. onderstepoort* O serum and in absorption tests removed all agglutinins from this serum. *S. blijdorp* O serum, absorbed with *S. onderstepoort*, gave no agglutination with *S. onderstepoort* and *S. blijdorp*. Therefore the culture belonged to O group H of the genus *Salmonella*; it contained the somatic antigens 1, 6, 14, 25.

The H-antigens were diphasic. Phase 1 was agglutinated to the titer of (1 : 5000) and removed in absorption tests all agglutinins from serum, derived from phase 1 of *S. cholerae suis* (c). Phase 2 was agglutinated by serum, prepared with phase 2 of *S. cholerae suis* (1,5).

In absorption tests not all agglutinins of phase 2 of *S. cholerae suis* were removed. The antigenic formula of the type examined was therefore 1, 6, 14, 25 : c : 1,5. Since similar types have not yet been described, the cultures were named *S. rotterdam* respectively *S. blijdorp*.

We are indebted to Dr. F. Kauffmann for confirmation of our findings.

Summary.

A description is given of two new *Salmonella* types. *S. rotterdam* with antigenic formula 1, 13, 22 : g, t : 1,5 was isolated from the feces of a tree-agame (*Calotes versicolor*). *S. blijdorp* with the antigenic formula 1, 6, 14, 25 : c : 1,5 was isolated from the feces of a cameleon (*Chameleon jacksoni*).

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CYTOCHEMISTRY AND SENSITIVITY OF A HUMAN AMNION CELL-LINE TO COXSACKIE A_{23} ($ECHO_9$) VIRUS

by

H. BEEUWKES[1]

(Received February 23, 1960).

Introduction.

Primary cultures of human amnion cells and continuous lines of these cells have been widely used for the propagation of viruses since the first report on the suitability of such cells was published by Zitcer, Fogh and Dunnebacke (1955). Amnion cells, however, are prepared in various ways.

Trypsinization, using 0.25% trypsin in buffer solution, varies in duration from 20 minutes to 6 hours (Weinstein *et al.*, 1956). Fogh and Lund (1957) used a dilution of trypsin of 1 in 400 (Difco 1/250) and versene in a dilution of 1 in 5000 in phosphate buffer solution without calcium and magnesium at pH 7.4 to 7.6. The number of cells varies from 300,000 to 3,000,000 per ml. The nutrient medium usually consists of 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 0.4% dextrose, Earle's or Hanks' balanced salt solution and the antibiotics penicillin, streptomycin and nystatin. Medium 199 may also be used. Lahelle (1956) used bovine amniotic fluid with either chick embryo extract or human placenta extract and either human or horse serum. Calf, cow or sheep (lamb) serum in concentrations varying from 10 to 20% may also be used. The amniotic membranes were received from 5 to 16 hours after delivery (Lahelle, 1957).

Lane and Marshall (1957) obtained a monolayer in 64% of the cultures within 7 days, and in another 10% after 7 days. Only

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[1]) With the technical assistance of the Reverend Sister Theocleta.

in 3% of 36 amniotic membranes tested no growth was obtained.

According to published data, the spectrum of viral activity in primary cultures is as follows: poliovirus all three types, adenovirus types 1 to 8, simian viruses 1 and 2, mumps, herpes simplex, measles, Coxsackie group A type 9, Coxsackie group B types 1 to 5, ECHO types 1, 3, 5 and 6, blue tongue, Newcastle disease, Western equine encephalitis.

Few data are available concerning the spectrum of viral activity in continuous lines of amnion cells. FERNANDES (1958) reported the sensitivity of an amnion cell-line to a number of viruses: blue tongue, adenovirus types 1, 2, 3, 4, 6 and 7, Newcastle disease, mumps, poliovirus all three types, herpes simplex and measles.

Since no data are available on possible changes in the sensitivity to viruses and the cytochemistry of continuous amnion cell-lines, a study of these questions has been undertaken.

MATERIALS AND METHODS.

Thirty minutes after it was received, amniotic membrane[1]) obtained from a caesarian section was washed with sterile Hanks' solution and spread out on sterile filter paper. Remnants of mucus and blood were removed, and after being washed again with Hanks' solution the membrane was cut into pieces and put in a bottle containing approximately 100 ml of trypsin solution and a magnetic mixer. The trypsin solution was prepared by dissolving Bacto trypsin 1/250 in a solution of:

NaCl	8.0 g	
KCl	0.2 g	
$Na_2HPO_4.2H_2O$	2.9 g	
KH_2PO_4	0.2 g	
bidistilled water	800 ml	
$CaCl_2$ (anhydrous)	0.1 g	dissolved in 100 ml bidistilled water
$MgCl_2.6H_2O$	0.1 g	dissolved in 100 ml bidistilled water
glucose	1 g	
bidistilled water ad	1000 ml	

The solution was sterilized by filtration through a Seitz EK disc. The bottle and the trypsin solution had been warmed to a temperature of 37° C. After 20 minutes of trypsinization, the turbid fluid

[1]) The amniotic membrane was kindly supplied by the obstetrical-gynaecological department, Head: Dr. TH. VAN SANTE.

was poured into a second bottle, which was placed with ice in a cup in the refrigerator at 5° C. This treatment was repeated twice. The cell suspension was then centrifuged for 5 minutes at 1000 r.p.m. and the sediment was twice washed with Hanks' solution. The washed cells were suspended in a nutrient medium consisting of Hanks' solution 60%, human, horse or calf serum 20%, lactalbumin hydrolysate (2.5%) 20% and penicillin 50 U, streptomycin 50 μg and nystatin 50 U per ml. We inoculated a dense suspension of ± 1,000,000 cells per ml and also another of ± 600,000 per ml, as our experience with concentrations of ± 500,000 cells per ml had been less satisfactory. From the 5th subculture, a combination of versene and a final dilution of 0.08% trypsin was used to remove the cells from the wall. The trypsin solution 0.25% was diluted with a solution consisting of

versene (tritriplex III Merck)	0.2 g
$CaCl_2$	8.0 g
KCl	0.2 g
$NaH_2PO_4.2H_2O$	1.15 g
KH_2PO_4	0.2 g
bidistilled water	1000 ml

After approximately 10 minutes, the cells became detached from the wall. The suspension contained many more loose cells and practically no clots when this solution was used instead of trypsin solution.

At the first transfer, the suspended cells were divided between 6 bottles, all of which contained the lactalbumin hydrolysate medium, two with 20% human serum, two with 20% horse serum and two with 20% calf serum. The medium was renewed after 1 day and again after 3 days. After 3 days incubation at 37° C. the outgrowth of cells was satisfactory and after 5 days the monolayer was practically complete. The cell cultures showed no morphological differences according to whether human or animal serum was used. After 5 days of incubation, the monolayer was transferred to 2 bottles, 20 tubes and 10 slides in Petri dishes, all containing the same nutrient medium. The early cell-lines produced in the media containing horse and calf serum failed to grow out, degenerated and were lost. The monolayer in the medium containing 20% human serum maintained itself satisfactorily, but showed little outgrowth in spite of the repeated renewal of the medium. A few cells, however, grew out to four islets having a diameter of approximately 1 mm.

They showed a distinct epithelioid character. The islets, when transferred to 7 tubes, yielded a good monolayer after 8 days, which was successfully transferred to 9 tubes. The 6th transfer produced a good monolayer in two bottles, and from then on, the cell-line grew without any difficulty. After the 7th transfer, the amount of human serum was reduced to 5%. At the time of writing, 24 transfers have been successfully made.

In order to obtain some understanding of the morphology and the metabolism of the amnion cells, the second transfer was carried out on coverslips of 24 × 32 mm in Petri dishes of a diameter of 6 cm and containing the usual nutrient medium. The cells were studied in preparations stained by the Papanicolau method, by the Sudan III reaction, the oxidase reaction with Janus green B (Edward Gurr) and alkaline phosphatase production according to the method of Gomori (Pearse, 1953).

The staining method of Papanicolau was used because it yields a particularly good picture of the nuclear structure.

The oxidase reaction was performed by adding 0.4 ml of a 1 in 1000 aqueous solution of Janus green to 4 ml of nutrient fluid; the fluid was suctioned off after 15 minutes at 37° C. and the preparation was covered with a coverslip. After this, the cells could be examined by dry system, phase contrast and oil immersion.

The alkaline phosphatase reaction was determined almost entirely according to the method of Gomori. The cell culture was rinsed in sterile saline, then in 4% neutral formalin solution and finally in sterile distilled water. The preparation was then incubated for 90 minutes at 37° C. in a solution consisting of 10 ml 2% beta sodium glycerophosphate, 10 ml 2% sodium diethyl barbiturate, 15 ml distilled water and 8 ml 2% calcium chloride (the latter must be stored in the refrigerator). Magnesium sulphate was not used, because it may cause diffusion resulting in non-specific pictures (Mellors, 1955).

After incubation, the preparation was first washed in running tap water and then in 2% cobalt citrate solution for 3 to 5 minutes. This solution must be stored in the refrigerator and regularly renewed. The preparation was then washed in distilled water, in equal parts of 10% ammonia and 10% thioacetamide for 1 to 2 minutes, and again for 20 minutes in running water. It was counterstained for 30 minutes with carmine-alum, washed once in distilled water, twice in alcohol 96% and twice in xylol. The preparation

should be placed on the slide with caedax. The formalin treatment is intended to kill the virus. We used 8 ml instead of 2 ml calcium-chloride to produce a stronger reaction.

Results.

Amnion cells are satisfactorily stained by the method of Papanicolau[1]) after 5 days. The nucleoli are usually spherical and show an even edge. There are generally 1 or 2, sometimes up to 4 nucleoli per nucleus. Sometimes they are rod-shaped. No mitoses are visible. The cytoplasm has a fine reticular structure. Irregularly-sized vacuoles are present in approximately 30% of the cells.

Stained preparations of the 7th transfer indicate an increase in the number of nucleoli, the average number being 2 or 3, sometimes 4 or 5. Most of the nuclei have an ovoid shape and many of them are dented and lobulated. Cells containing 2 to 11 nuclei and large cells with one large nucleus showing little differentiation may be observed. Mitosis is observed in approximately 1% of the cells. The general appearance of the densely grown 5 days' old culture is irregular and the cells are often elongated.

Whereas the second transfer showed a strong Sudan reaction, the cells containing orange globules of varying size, approximately 40% of the cells of the 7th transfer contain an average of 10 small orange granules. Vital staining of the second transfer with Janus green B reveals blue-stained mitochondria and the nucleoli are stained a pale blue. There is a bright unstained area round the edge of the cell. The 7th transfer shows an intense reaction with Janus green B. The nucleoli are dark-blue, the nucleus is light-blue. The cytoplasm has a regular structure in which blue-stained mitochondria are visible. The 7th transfer seems to show a markedly stronger oxidase reaction.

A similar difference in intensity is also observed in the activity of alkaline phosphomono-esterase. The second transfer showed a negative reaction in contrast to the 7th transfer in which almost all the cells show a dark-brown precipitate of cobalt sulphide in the cytoplasm, the cells in mitosis especially being stained intensely dark-brown.

The propagation of one of the strains of $ECHO_9$ (Coxsackie A_{23})

[1]) I am indebted to Dr. H. O. van der Zalm for the Papanicolau stains and his instructions in cytochemistry.

virus isolated in the Netherlands[1]) has been studied in various transfers of the amnion cells. This strain had been isolated and passed through monkey kidney cultures. 0.1 ml of a virus dilution was inoculated into 3 tubes of the second subculture of amnion cells after the monolayer had been twice washed with Hanks' solution. The same medium containing 5% calf serum was used as nutrient fluid for the propagation of the virus. The virus produced a complete cytopathogenic effect after 5 days in a dilution of 10^{-6}. No further titration has been performed.

In the 4th transfer of amnion cells, the virus was inoculated into 4 tubes in a dilution of 10^{-3}. The 4th transfer, originating from 4 cell islets of the preceding transfer, yielded just enough cells for 7 tubes. After one week no cytopathogenic effect was observed. Repeated inoculation of cells of the 7th transfer with 0.15 ml of virus-containing fluid in dilutions of $10^{-0.5}$, 10^{-1}, 10^{-2} and 10^{-3} again demonstrated complete insensitivity of the cell-line to this virus. Simultaneous titration in monkey kidney culture resulted in a complete cytopathogenic effect in the dilution of 10^{-5} and a partial cytopathogenic effect in a dilution of 10^{-6}. The control culture showed an intact monolayer. It seems, therefore, that amnion cells become insensitive to this virus in the 4th, and probably already in the 3rd transfer.

When stained by the Papanicolau method, amnion cells in which $ECHO_9$ (Coxsackie A_{23}) virus propagates show the following changes: the nucleoli disappear and the nucleus shows a grossly granular aspect, which ultimately changes into pycnosis via an intermediate stage showing larger lumps; a number of nuclei shrink and show a striated pattern. At first, there are no changes of the cytoplasm; the cell may, however, shrink, in which case the cytoplasm is stained strongly green-blue.

Discussion.

This investigation shows that amnion cells in primary culture are sensitive to ECHO virus type 9. The 4th transfer, and probably the 3rd, however, are insensitive to this virus. The Sudan reaction is markedly positive during the 2nd transfer, in contrast to the 4th transfer. Conversely, the alkaline phosphomono-esterase reaction

[1]) Obtained from the Laboratory for Microbiology of the University of Leiden (Prof. Dr. J. D. Verlinde).

is initially negative and later positive. In later transfers, the cytochrome oxidase reaction is markedly stronger. This difference, however, is difficult to assess, because there are too many factors that might give rise to a difference in reaction.

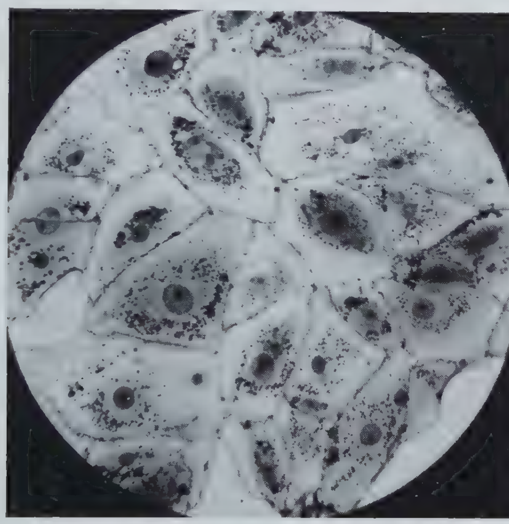

Fig. 1. Amnion cells, 5 days old 2nd passage, stain Sudan III [1]).

Morphologically, the 4th and further transfers are qualitatively distinct from the 1st and 2nd transfers (figs. 1 and 2). The typically monotonous picture of angular cells with a relatively smaller nucleus, which at once suggests an intact amniotic membrane, changes into an apparently active chaotic cell pattern from the 3rd transfer. The observations described allow comparison with the investigation of PULVERTAFT, DAVIES, WEISE and WILKINSON (1959).

These writers report that thyroid cells in primary culture are sensitive to ECHO virus, types 6 and 9. The cell-line becomes insensitive after a few transfers. The transformed cell-line grows out from a few cell islets. The primary culture, in contrast to the transferred cells, is capable of synthesizing organic iodine compound.

Thus it is probable that the amnion cells retain their specific

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1) Photographs by Dr. H. O. VAN DER ZALM.

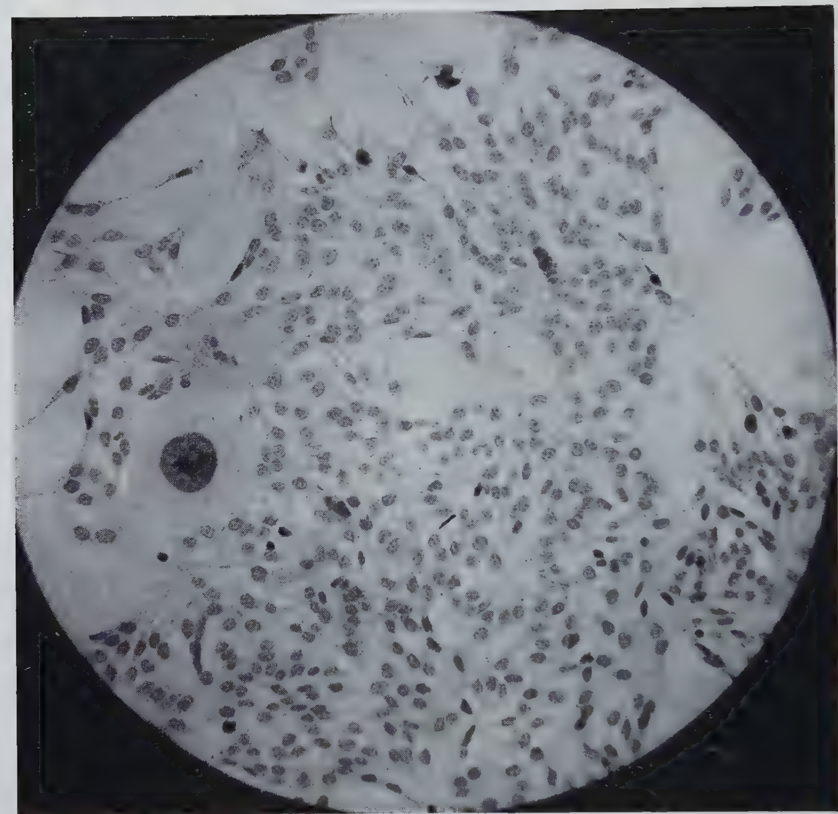

Fig. 2. Amnion cells, 7th passage, stain Sudan III.

properties during primary and secondary culture and remain sensitive to $ECHO_9$ virus and a number of other viruses. In the adaptation in vitro only those cells of the population which are able to create a changed metabolism and growth pattern maintain themselves. This is accompanied by a loss of specificity and sensitivity to $ECHO_9$ virus. The strong Sudan III reaction of the 2nd transfer and the absence of any alkaline phosphatase reaction may suggest a change in metabolism. On the other hand, it is possible that during the 2nd transfer only fatty compounds can be absorbed from the nutrient medium to an increased extent. The absence of the alkaline phosphatase reaction may, however, also be explained by the enzyme being present, but in a quantitatively insufficient degree.

Further study is required to explain the difference in metabolism between primary transfers and the continuous cell-line. Properties might be found to exist opposing the growth of the ECHO virus.

Summary.

A description is given of the isolation of an amnion cell-line. The morphological and cytochemical changes of the 2nd and 4th transfers are compared. It is shown that the 4th transfer becomes insensitive to ECHO virus type 9. The growth pattern changes also, while the Sudan reaction, after having initially been markedly positive, becomes weakly positive. The alkaline phosphatase reaction, negative in the beginning, becomes positive in the 4th transfer.

The amnion cells lose their specific characteristics during the adaptation.

It is possible that we are dealing here with a general adaptation phenomenon.

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A STUDY OF THE INFLUENCE OF POLYMYXINE COMBINED WITH CHLORAMPHENICOL ON *SALMONELLA* IN VITRO AND IN VIVO IN CARRIERS OF *S. PARATYPHI* B

by

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Introduction.

It is of the greatest importance from the epidemiological point of view, that *Salmonella* present in the feces of healthy persons be eliminated, and it is therefore not surprising that many antibiotics, singly or in combinations, have been investigated in vitro in an attempt to arrive at an effective in vivo therapy. In 1956, Manten and de Nooy reported a study from which they concluded that the combination of 5 μg/ml polymyxine B and 10 μg ml chloramphenicol in vitro has a synergistic action on many Salmonellae, notably 17 strains of *S. typhosa*, 20 of *S. paratyphi* B, 16 of *S. bareilly*, *S. manhattan*, *S. dublin*, *S. heidelberg* and various other strains. These authors emphasized that in vivo experiments are required in order to confirm the observed synergism with bactericidal result.

In 1959 the same authors reported that the association of bacteriostatic concentrations of chloramphenicol and polymyxine has an intensely bactericidal effect. Addition of chloramphenicol to bactericidal concentrations of polymyxine, on the other hand, did not enhance the bactericidal effect.

Finally, these authors have mentioned that the results they have obtained in recent carriers justify a certain degree of optimism; details are not given.

We have considered it of interest to report the results of a study of the efficacy of a combination of chloramphenicol and polymyxine

in a number of *S. paratyphi* B excreters. We will also report a study of the effects of polymyxine, of chloramphenicol and of their combination on *S. paratyphi* B and on *S. typhimurium* in vitro.

Clinical investigation.

This investigation was rendered possible by an explosion of paratyphoid B in nursing personnel and servant girls. The patients were treated with 2 g chloramphenicol per day for 5 days [1]). In the majority of the patients, an abatement of the fever was seen on the second day of the treatment. Diarrhoea was not observed. The fever was of the inverse type; most cases showed a typhoid form. *S. paratyphi* B [2]) could be isolated from the feces of all patients before and after the treatment. In order to assess the spread of the epidemic, all members of the nursing personnel, the kitchen staff and the servant girls were tested for the presence of *S. paratyphi* B in the feces.

After repeated examination of all servant girls and nurses concerned, a group of 42 excreters was found, consisting of 12 servant girls and 30 nurses. Both these groups were divided into 3 subgroups: one control group, one group treated with 2 g chloramphenicol and 150 mg polymyxine per day and one group treated with 150 mg polymyxine per day. The treatment was continued for one week.

A. Group of servant girls.

On March 31, 1959, the feces of 12 girls were positive for *S. paratyphi* B.

a. 4 girls received polymyxine and chloramphenicol from April 3 10.
b. 4 girls received polymyxine from April 3–10.
c. 4 girls were given no antibiotics.

The first bacteriological examination was carried out on April 13. (The bacteriological tests were carried out by the methods habitually used in the Regional Public Health Laboratories, and are not described in detail).

[1]) We thank Chas. Pfizer and Co. for delivering polymyxine and chloramphenicol.

[2]) Phage typing was performed at the Rijks Instituut voor de Volksgezondheid, Utrecht. Strains of *S. paratyphi* B proved to be phage type I.

Results.

a. Group treated with polymyxine and chloramphenicol.
The feces were tested 8 times after treatment.
Two girls were positive once after treatment. Two girls were positive twice after treatment.
In all 4 girls, therefore, it proved still possible to isolate *S. paratyphi* B from the feces after combined treatment with polymyxine and chloramphenicol. After May 2, finally, 3 girls were negative, and the 4th became negative after a culture on May 6 had been the last to be positive.

b. Group treated with polymyxine.
The feces were tested 10 times after treatment.
Two girls were positive once after treatment. One girl was positive 7 times after treatment. One girl was negative after treatment.
In 3 girls, therefore, it proved still possible to isolate *S. paratyphi* B after treatment with polymyxine. With the exception of one girl who was still positive on May 9, the girls in this group became negative in the period between March 31 and April 16.

c. Control group.
The control tests were carried out after March 31.
One girl was positive once, on April 7 (7 cultures made).
One girl was positive twice, on April 5 and April 11 (10 cultures made).
One girl was positive once, on April 1 (7 cultures made).
One girl was positive 3 times, on April 2, April 10 and May 2 (9 cultures made).
With the exception, therefore, of one girl who was still positive on May 2, the girls of the control group became negative in the period between April 1 and April 14.

B. Group of nurses.

On April 12, the feces of 30 nurses were found to be positive for *S. paratyphi* B.

a. Ten nurses received polymyxine and chloramphenicol between April 13 and April 20.
b. Ten nurses received polymyxine between April 13 and April 20.
c. Ten nurses were given no antibiotics.

The first bacteriological control test was carried out on April 24.

R e s u l t s.

a. Group treated with polymyxine and chloramphenicol.
The feces were tested 5–6 times after treatment.
Four nurses were positive once after treatment. One nurse was positive twice after treatment. Five nurses were negative after treatment.
In 5 nurses, therefore, it was still possible to isolate *S. paratyphi* B from the feces after treatment with the two combined antibiotics. After May 6, all nurses in this group had become negative.

b. Group treated with polymyxine.
The feces were tested 7 times after treatment.
Nine nurses were negative after treatment. One nurse was positive once after treatment. After April 24, all had become negative.

c. Control group.
Seven nurses were negative at least 3 times after April 20.
Three nurses were positive once after April 20, *viz.*, on April 22, April 28 and May 5, respectively. After May 5, all of them had become negative.

The above findings can be summed up as follows: Both control groups showed a tendency to become negative. There is no distinct difference between the results in the control groups, the groups treated with polymyxine and chloramphenicol combined and the groups treated with polymyxine alone. In 9 out of 14 girls treated with the combination of antibiotics, *S. paratyphi* B could still be isolated from the feces after treatment.

These results will be discussed following a report of the investigation into the activity of polymyxine, of chloramphenicol and of their combination on Salmonellae in vitro.

In vitro investigation.

In order to collect some information concerning the sensitivity of a number of strains of *S. paratyphi* B in vitro, a study was made of 15 strains isolated in the course of the Sittard epidemic. In this study 5 strains of *S. typhimurium* were also included.

In this investigation we used the replica technique. This technique has been described for the first time by Lederberg and Lederberg (1952) and has also been used by Manten and de Nooy (1956)

in their study of the synergistic effects of a number of antibiotics.

A study was made not only of the synergistic action of polymyxine and chloramphenicol but also of the activity of polymyxine B. After oral administration the latter antibiotic is practically not absorbed by the intestinal wall (Walter and Heilmeijer, 1954). It is therefore possible to obtain high concentrations in the intestine, and the risk of undesirable side effects is very small, in contrast to what is observed after intramuscular administration. Polymyxine cannot penetrate into the tissues after oral administration.

Methods.

With the aid of the paper-disk method, the sensitivity to the two antibiotics was determined for different concentrations and also for differently proportioned concentrations.

With this method it is not possible, however, without determination of the polymyxine concentration in the medium at different distances from the disk, to obtain exact data concerning the strength of the polymyxine concentration which just inhibits bacterial growth; the method might, therefore, be called "semi-quantitative". Assuming that the diffusion of the readily soluble polymyxine occurs at the same rate as that of the antibiotics of the Gist- en Spiritusfabriek, Delft, that can be obtained commercially for this purpose, the amount of polymyxine applied to the disk approximately corresponds to the number of μg per ml of the medium at a distance of approximately 6 mm from the centre of the disk. When the diameter of the inhibition zone brought about by a disk with 5 μg polymyxine is approximately 12 mm, then 5 μg of polymyxine per ml is the concentration that just inhibits bacterial growth.

The chloramphenicol used in these experiments belonged to the set of antibiotics of the Gist- en Spiritusfabriek, Delft. When a disk with 10 μg chloramphenicol gave an inhibition zone of 10 mm, then the concentration of chloramphenicol which just inhibited bacterial growth was 15μg per ml.

A dilution of a broth culture of the strain to be studied was applied to a plate and the disks containing the antibiotics were placed on these plates; the diameters of the inhibition zones were measured after 20 hours' incubation. Subsequently, the replica plates were inoculated with velvet-covered stamps. On the next day,

the bacterial growth, and the inhibition zone on the replica plate were assessed according to the following criteria:

C (bactericidal effect): < 5 colonies within inhibition zone.
P (partial bactericidal effect): 5–10 colonies within inhibition zone.
S (bacteriostatic effect): > 10 colonies within inhibition zone.
C/S: Bacteriostatic effect at the periphery of the inhibition zone, and bactericidal effect in the centre of the inhibition zone.

Results.

Table 1 lists the mean values of the diameters of the inhibition zones in a practically confluent growth of colonies of *S. paratyphi* B strains on blood-agar plates caused by antibiotics applied in different concentrations on disks. The sensitivity of bacteria to polymyxine in concentrations lower than that bringing about an inhibition zone with a diameter of 7 mm cannot be determined with this method.

TABLE 1.

Bacteriostatic and bactericidal action of polymyxine, of chloramphenicol and of the combination of the two on 15 strains of *S. paratyphi* B (mean values).

antibiotic μg/disk		C/S	diam. in mm	synergism
polymyxine	chloramphenicol			
0.5		—	—	
1.0		C	7.3	
5.0		C	10.0	
10.0		C	11.0	
25.0		C	13.6	
50.0		P	14.3	
	10.0	S	9.0	
	25.0	S	15.0	
0.5	10.0	C/S	7.3	
1.0	10.0	C/S	8.7	
5.0	10.0	C	11.3	+
10.0	10.0	P	12.3	
25.0	10.0	C	14.5	
50.0	10.0	C	15.3	
0.5	25.0	C/S	14.0	
1.0	25.0	C/S	14.0	
5.0	25.0	C	14.7	+
10.0	25.0	C	14.9	+
25.0	25.0	P	15.8	
50.0	25.0	P	15.7	

Table 2 lists the mean values for 5 strains of *S. typhimurium*, determined in an identical way. It can be seen that polymyxine has a bactericidal action on strains of *S. typhimurium* as well as *S. paratyphi* B.

TABLE 2.

Bacteriostatic and bactericidal action of polymyxine, of chloramphenicol and of the combination of the two on 5 strains of *S. typhimurium* (mean values).

antibiotic μg/disk		C/S	diam. in mm	synergism
polymyxine	chloramphenicol			
0.5		—	—	
1.0		C	7.1	
5.0		C	10.0	
10.0		C	11.6	
25.0		C	13.6	
50.0		P	15.0	
	10.0	S	17.8	
	25.0	S	21.2	
0.5	10.0	C/S	13.4	
1.0	10.0	C/S	15.3	
5.0	10.0	C	15.6	+
10.0	10.0	C	16.0	+
25.0	10.0	C	17.2	+
50.0	10.0	C	18.4	+
0.5	25.0	P/S	19.4	
1.0	25.0	P/S	19.2	
5.0	25.0	C/S	19.2	
10.0	25.0	C	20.0	+
25.0	25.0	C	20.0	+
50.0	25.0	C	20.4	+

It is a remarkable finding that only a partial bactericidal effect was brought about by polymyxine 50 μg/disk and by higher concentrations of polymyxine in combination with chloramphenicol in high concentrations in 15 out of 20 strains. We have been unable to find an explanation of this phenomenon.

Tables 1 and 2 prove that synergism exists between polymyxine and chloramphenicol. Because of the pronounced sensitivity to chloramphenicol of 4 out of 5 *S. typhimurium* strains the synergistic effect emerges even more clearly with these strains than with the *S. paratyphi* B strains, as a consequence of the great differences

in diameter of the inhibition zones caused by polymyxine and by chloramphenicol, respectively.

Discussion.

Although the methods used in the bacteriological study were different from those applied by MANTEN and DE NOOY (1956) the results on the whole are in agreement with the findings obtained by these authors. For certain proportions of the concentrations of polymyxine and chloramphenicol a synergism could be demonstrated in vitro. In contrast to an observation by MANTEN and DE NOOY (1959), this synergism was also evident for higher concentrations of polymyxine (5 and 10 μg ml). However, these concentrations cannot be judged accurately with the paper disk method. Possibly, the differences with the findings of MANTEN and DE NOOY are to be attributed to the insufficient diffusion in agar.

The results of the clinical study are not completely comparable with those of the studies by MANTEN and DE NOOY in recent carriers of Salmonellae. On one hand, we were in the favourable position that we could study a homogeneous group of excreters; on the other hand, the efficacy of the combination of antibiotic was more difficult to study in vivo because this group of excreting carriers were examined immediately after the outbreak occurred. As observed in the control groups, many of them became negative spontaneously.

The question whether combined treatment with polymyxine and chloramphenicol is indicated for recent and chronic excreters of *Salmonella* cannot be answered on the basis of the findings in the study reported above. Although MANTEN and DE NOOY in some cases saw good results of this treatment in recent excreters, we cannot ignore the fact that after the combined treatment *S. paratyphi* B could still be isolated in 9 out of 14 girls. Isolation after treatment was also possible in 4 out of 14 girls who had been treated with polymyxine alone.

The discrepancy between the results of in vitro studies and the clinical results is perhaps to be attributed to the fact that polymyxine does not penetrate into the biliary ducts and the gall bladder, which are well known as foci in *Salmonella* carriers. The varying results of the clinical application of the combination of antibiotics might be explained by the same fact.

Summary.

A study was made of the effect of polymyxine and of chloramphenicol on Salmonellae in vitro, and also in vivo in a number of carriers of *S. paratyphi* B.

The findings of other investigators concerning synergistic action of polymyxine and chloramphenicol in vitro could be confirmed.

Excreters who had had a course of treatment with polymyxine and chloramphenicol in most of the cases were still found positive one or more times for *S. paratyphi* B in the feces. Most excreters became negative spontaneously.

The question whether combined antibiotic treatment is indicated for chronic excreters of *Salmonella* is not answered. The differences between the results of in vitro and in vivo experiments, and the divergences in the results obtained after in vivo application by these and other authors are possibly to be atributed to an insufficient or absent penetration of polymyxine into the biliary ducts and the gall bladder.

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ETUDE IMMUNOLOGIQUE DE SERUMS DE MOUTON ANTIDIPHTERIQUES

II. ETUDE SUR LES ANTICORPS NEUTRALISANTS ET NON PRECIPITANTS [1])

par

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avec la collaboration technique de Mademoiselle O. Bepoldin et Monsieur W. J. van Rhee

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Introduction.

Dans une publication antérieure, nous avons décrit les caractères physico-chimiques et immunologiques des divers anticorps antidiphtériques présents dans le sérum de moutons hyperimmunisés (Relyveld *et al.*, 1959a). Nous avons pu montrer que les anticorps qui neutralisent l'action toxique de la toxine diphtérique sont, chez ces animaux, des anticorps coprécipitants. Nous apportons ici des résultats complémentaires relatifs à l'isolement de ces anticorps coprécipitants et à l'étude de leurs propriétés.

Matériel et méthodes.

Les techniques employées (précipitation spécifique en gélose, immuno-électrophorèse, précipitation quantitative etc...) ont été décrites précédemment (Relyveld *et al.*, 1959a, 1959b). Nous signalerons donc seulement, le cas échéant, les modifications que nous avons dû leur apporter.

Les sérums étudiés étaient des mélanges de nombreuses saignées effectuées chez divers animaux hyperimmunisés.

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[1]) Première partie: Antonie van Leeuwenhoek **25**, 369, 1959.

Trois lots distincts ont été employés. Ils seront désignés par M 1, M 2 et M 3.

Leurs propriétés sont rapportées ci-dessous:

	U.I./ml	U.F./ml
M 1	650	235
M 2	650	220
M 3	550	85

La purification de ces anticorps par digestion enzymatique a été mise au point par l'un d'entre nous (van Triet, 1959). Nous nous sommes basés sur cette technique pour digérer les précipités spécifiques obtenus à l'optimum de floculation, ainsi que leurs surnageants.

La toxine employée pour ces études était une préparation cristallisée titrant 3020 UF/mg N. La technique de marquage à l'iode 131 décrite pour la toxine, a été employée dans les mêmes conditions pour préparer des anticorps radioactifs.

Résultats.

1) Préparation des anticorps neutralisants non précipitants.

Les surnageants de la précipitation quantitative effectuée dans les conditions correspondant à l'optimum de floculation (floculation initiale) ne contiennent plus d'anticorps précipitants. Ils présentent encore une teneur élevée en anticorps neutralisants ; nous avons donc soumis à la digestion pepsique d'une part le précipité spécifique, d'autre part le surnageant correspondant.

Les surnageants ont été digérés selon la technique décrite par l'un d'entre nous non modifiée (van Triet, 1959).

Pour les précipités spécifiques, nous avons dû modifier certains points de la méthode. Nous avons déterminé d'abord la durée optimale de digestion.

A 250 ml de sérum brut M 1, titrant 650 Lr et 235 UF/ml, ont été ajouté 14,7 ml d'une solution d'anatoxine partiellement purifiée (2025 UF/mgN) contenant 4000 UF par ml; le pH est ajusté à 6,5. Après floculation à 45° C., le surnageant (M 1–S) et le précipité (M 1–P) sont séparés par centrifugation. Le précipité est lavé et

remis en suspension dans 300 ml d'eau physiologique; après avoir ajouté 1 g de pepsine brute (Zimmerman and Co) et ajusté le pH à 3,2, la solution est répartie en 5 échantillons de 60 ml, qui sont alors placés au bain-marie à 30° C. pendant des temps différents. Après digestion, le pH est ajusté à 7.

Le tableau 1 montre les titres de ces préparations digérées (P.D.), en fonction de la durée de digestion, ainsi que celui du surnageant brut (S) et celui du surnageant digéré et purifié par relargage (S.D.).

TABLEAU 1.

Préparation	Durée de digestion	Titres "in vivo" U.I./ml	Titres "in vitro" UF/ml
M 1	—	650	235
M 1–S	—	250	0
M 1–S.D.	1 h.	650	0
M 1–P.D.	1 h.	100	59
,,	2 h.	100	59
,,	3 h.	100	56
,,	4 h.	100	54
,,	5 h.	100	50

M 1–S = surnageant de la floculation initiale.
M 1–S.D. = surnageant de la floculation initiale digéré, purifié et concentré par ultrafiltration (volume final: volume initial = 1 : 7).
M 1–P.D. = précipité remis en suspension et digéré.

Nous avons adopté 1 heure comme temps de digestion; nous décrirons brièvement la façon d'obtenir les deux séries de préparations que nous avons employées pour nos expériences.

A 1500 ml de sérum de mouton brut M2, dont les caractères sont indiqués dans le tableau 2, nous avons ajouté 82,5 ml d'anatoxine (4000 UF/ml, 2025 UF/mgN) pH = 6,5. Centrifugation après 30 minutes à 30° C.

Volume du surnageant (M 2–S) : 1300 ml, et du précipité (M 2–P) après lavage avec une solution physiologique : 280 ml.

Le précipité a été suspendu dans un volume final de 1800 ml de solution physiologique. Après avoir ajusté le pH à 3,2 et ajouté 5 g de pepsine, la solution a été digérée pendant 60 minutes à 30° C. Après digestion, on a ajouté 2,5 g de $Na_4P_2O_7$, 261 g de $(NH_4)_2SO_4$ (= 14,5%) et après ajustage du pH à 4,5, 5 ml de toluène.

Le mélange est chauffé pendant 60 minutes à 50° C. et le précipité

formé, éliminé par filtration. Le filtrat (1900 ml) est additionné de 3,8 g de $Na_4P_2O_7$ et 171 g de $(NH_4)_2SO_4$ (= 9%) et le pH ajusté à 5,3. Le précipité formé M 2–P.D. a été dialysé contre de l'eau courante et ensuite ultrafiltré jusqu' à un volume de 110 ml.

Au surnageant M 2–S (volume 1300 ml), additionné de 2600 ml d'eau et ajusté à pH 3,2, ont été ajoutés 7,8 g de pepsine (le pH monte à 3,4). Digestion pendant 35 minutes à 27° C.

Après digestion, on a ajouté 7,8 g de $Na_4P_2O_7$ et 565,5 g de $(NH_4)_2SO_4$ (= 14,5%), et, après ajustage du pH à 4,5, 3,9 ml de toluène. Ensuite, chauffage pendant 65 minutes à 50° C. et filtration.

Au filtrat (3900 ml), on ajoute de nouveau 7,8 g de $Na_4P_2O_7$ et 253,5 g de $(NH_4)_2SO_4$ (= 6,5%) pH = 5,3. Le précipité M2–S.D. est dialysé et ultrafiltré jusqu' à obtention d'un volume de 200 ml. On trouvera les caractères des fractions dans le tableau 2.

Le sérum brut M3 est un mélange de 70 saignées à 70 ml. Un mélange de 3000 ml de M3 et de l'anatoxine à l'optimum de floculation reste 2 heures à 37° C. et 2 jours à 0 °C. (conditions de l'établissement des courbes de précipitation quantitative). Le précipité formé (360 ml), est lavé et homogénéisé dans 1800 ml de solution physiologique. La digestion se fait pendant 60 minutes à pH = 3,2 après addition de 5 g de pepsine.

Après digestion, on ajoute 2,5 g de $Na_4P_2O_7$ et 261 g de $(NH_4)_2SO_4$ (= 14,5%).

Après ajustage du pH à 4,5, on ajoute 5 ml de toluène, suivi de chauffage pendant 65 minutes à 50° C. Le précipité est éliminé par filtration, et au surnageant (volume 1800 ml) l'on ajoute 2 g de $Na_4P_2O_7$ et 162 g de $(NH_4)_2SO_4$ (= 9%).

Le dernier précipité ayant, après dialyse, un volume de 260 ml, on lui ajoute : NaCl 0,85%; éther 0,4%; tricrésol 0,4%. Cette préparation M3–P.D. est concentrée par ultrafiltration jusqu' à un volume final de 86 ml.

A 2700 ml de surnageant M3–S dilué au 1/3 avec de l'eau, ont été ajoutés 16,2 g de pepsine. Le pH était préalablement ajusté à 3,4. Après digestion pendant 35 minutes à 27° C. et addition de : 16,2 g de $Na_4P_2O_7$, 1174,5 g de $(NH_4)_2SO_4$ (pH = 4,5) et 8,1 ml de toluène; le mélange est chauffé pendant 65 minutes à 50° C. Le précipité qui se forme est enlevé par filtration. Au surnageant, dont le volume égale 8 litres, on a ajouté 16 g de $Na_4P_2O_7$ et 520 g de $(NH_4)_2SO_4$ (6,5%); pH = 5,3.

TABLEAU 2.

Préparation	Titres par ml "in vivo"	Titres par ml "in vitro"	Lr/Lf	mg N/ml	Lr/mg N	UF/mg N	Rend. Lr %	Rend. UF %[1])
M2	650	220	2,95	12,23	53,2	18,0	—	—
M2–S	250	n.f.p.[2])	—	10,54	23,7	—	33,4	—
M2–S.D.	480	n.f.p.	—	8,42	57	—	9,85	—
M2–P.D.	850	444	1,91	2,74	310	162	9,6	14,8

[1]) Rendements calculés par rapport à la fraction M2.
[2]) n.f.p. ne flocule pas dans des conditions habituelles : 4 ml d'anatoxine ou toxine à 20 UF/ml; sérum à volume variable dilué de façon à faire environ 200 UF/ml.

TABLEAU 3.

Préparation	Titres par ml "in vivo"	Titres par ml "in vitro"	Lr/Lf	mg N/ml	Lr/mg N	UF/mg N	Rend. Lr %	Rend. UF %[1])
M3	550	85	6,47	12,04	45,7	7,06	—	—
M3–S	315	< 1	—	10,44	30,2	—	55,4	—
M3–S.D.	2250	< 4	—	28	80,3	—	36,6	—
M3–P.D.	825	500	1,65	2,36	350	212	4,3	16,9

[1]) Rendements calculés par rapport à la fraction M3.

Le précipité dialysé (1400 ml) est additionné de: NaCl 0,85%, tricrésol 0,4%; éther 0,4%, et ultrafiltré jusqu' au volume de 250 ml.

Les caractères des fractions sont présentés dans le tableau 3.

2) Etude de la réaction de précipitation quantitative.

Le précipité qui a servi à l'obtention des préparations d'anticorps précipitants se forme à l'optimum de floculation de la réaction "anatoxine-antitoxine". Nous avons donc d'abord étudié la réaction quantitative avec une anatoxine; les études antérieures avaient toujours été faites avec une toxine brute ou pure.

Nous avons étudié la réaction entre le sérum M2 et l'anatoxine partiellement purifiée (2025 UF/mg N) qui a servi à l'obtention des sous-fractions, ainsi que la réaction entre ce sérum et une toxine pure.

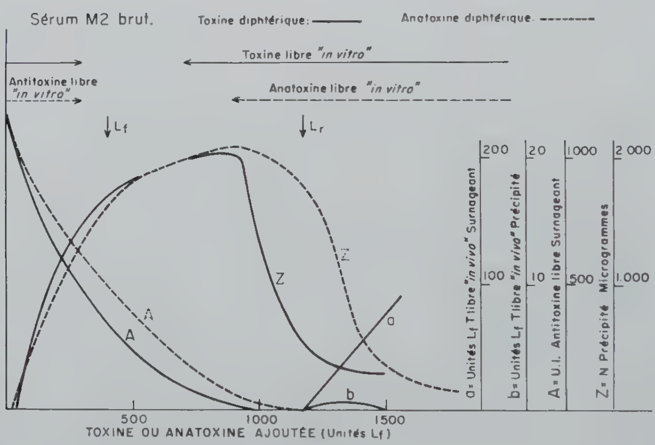

Fig. 1.

La fig. 1 montre que les 2 courbes sont du même type, quoiqu'elles présentent certaines différences. Pour la première partie de la courbe, les quantités d'azote précipitées avec la toxine sont légèrement supérieures à celles précipitées avec l'anatoxine. La courbe de neutralisation obtenue à partir des titres "in vivo" indique que la toxine pure possède un pouvoir de combinaison supérieur à celui de cette préparation d'anatoxine. On trouve aussi de la toxine libre détectable "in vitro" dans les surnageants bien avant que n'apparaisse de l'anatoxine libre.

L'azote précipitable dans la partie descendante de la courbe est plus élevé avec l'anatoxine et met en évidence l'influence des systèmes "accessoires" réagissant dans cette région de la courbe.

Au point Lf, il n'y a plus d'antitoxine libre détectable "in vitro" dans le surnageant, tandis qu'on y retrouve encore environ 40% des anticorps neutralisants, raison pour laquelle nous nous sommes servis de ce surnageant et du précipité correspondant pour préparer les sous-fractions.

La courbe de précipitation quantitative obtenue avec la fraction M2–P.D. et une toxine pure est présentée dans la fig. 2.

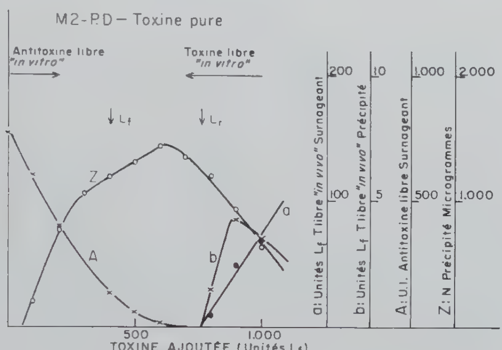

Fig. 2.

Nous voyons que la solubilité en excès d'anticorps est, dans ce cas, plus prononcée qu'avec le sérum total digéré. Les anticorps de mouton digérés (γ^1–S) sont donc bien de "type intermédiaire" (Relyveld, 1959a). Cette courbe montre de nouveau que le point Lr se trouve dans la partie descendante de la courbe, à droite du point Lf. Remarquons aussi la toxicité des précipités après le point Lr.

La figure 3 représente la réaction avec la fraction M2–S.D. Il y a formation d'une très faible quantité de précipité dans la zone où la quantité de toxine ajoutée est peu élevée. Cette préparation contient donc encore des anticorps précipitants. La courbe de neutralisation de l'antitoxine est à peu près une droite. On trouve de la toxine libre "in vitro" après avoir ajouté 200 UF de toxine au mélange contenant 800 UI, le volume final étant comme toujours égal à 5 ml.

Nous avons pensé que le temps de précipitation respecté pour l'élimination des anticorps précipitants du sérum brut était insuffi-

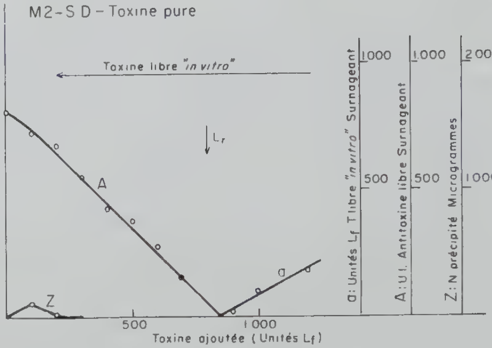

Fig. 3.

sant et qu'il restait encore dans le surnageant des complexes "toxine-antitoxine précipitants" solubles.

C'est pourquoi nous avons prolongé le temps de contact pour préparer le précipité spécifique à partir du sérum M3 : 2 heures à 37° C. et 48 heures à 0° C.

La figure 4 montre qu'il n'y a plus de précipité avec la fraction M3–S.D. La réaction toxine-antitoxine diphtérique, et plus précisément, la neutralisation des deux constituants, se fait sans formation de précipité.

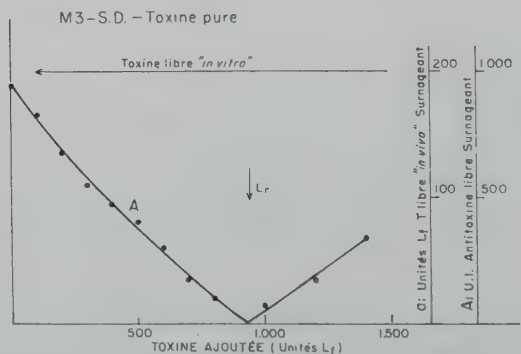

Fig. 4.

La floculation est, d'après ces résultats, un phénomène distinct de la neutralisation de la toxine par l'antitoxine.

Les complexes solubles "toxine-anticorps non précipitants" peuvent réagir avec des anticorps précipitants. On trouve de la toxine libre "in vitro" dans toute la zone d'excès d'anticorps.

Nous avons également étudié l'influence des anticorps neutralisants non précipitants sur des mélanges précipitants.

Dans ce but, nous avons eu recours à des mélanges artificiels contenant des quantités différentes d'anticorps non précipitants.

La figure 5 montre les courbes de deux mélanges M2–P.D. + M2–S.D. ayant des valeurs Lr/Lf = 2,89 et 3,90. Nous portons en pointillé sur la même figure la courbe du précipité digéré M2–P.D. Toutes les courbes ont été faites avec une toxine titrant 3020 UF/mg N.

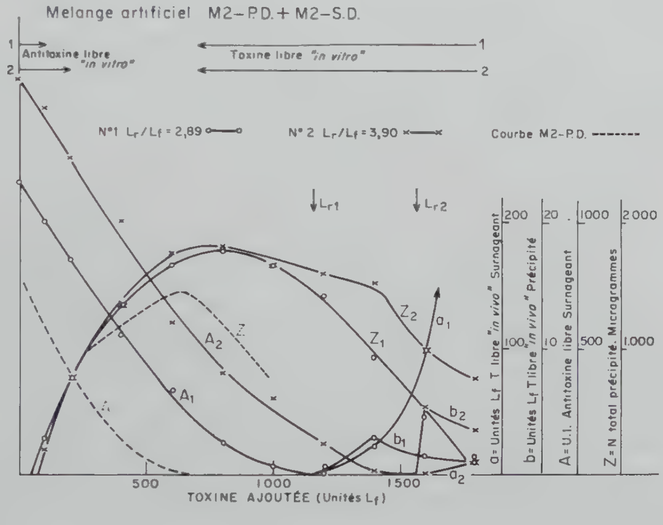

Fig. 5.

Les trois courbes sont faites avec la même quantité d'anticorps, précipitants (correspondant à 400 UF de la préparation M2–P.D.) additionnée de quantités différentes de la préparation non précipitante (M2–S.D.) Les conclusions suivantes peuvent être tirées de l'examen de ces courbes.

1) l'N total précipité augmenté par adjonction de la préparation non précipitante. Les anticorps de celle-ci sont donc coprécipitables.

2) Les anticorps précipitants ne peuvent coprécipiter qu'une quantité limitée d'anticorps non précipitants.

l'Azote anticorps précipité est en effet le même pour les 2 mélanges de rapports Lr/Lf respectifs, 2,89 et 3,90.

Les observations antérieurement rapportées pour les titrages "in vivo" des anticorps libres dans les surnageants, la présence de

toxine libre dans les précipités et les surnageants après le point Lr, ainsi que la position du point Lr à droite du point Lf restent toujours valables.

Nous avons néanmoins remarqué qu'après un séjour prolongé des surnageants des mélanges à 0° C., il se formait encore une faible quantité de précipité pour les points situés après le maximum. Ce n'était pas le cas pour le sérum brut ou la fraction P.D.

Nous avons donc prolongé le séjour à 37° C. des fractions et mélanges de la préparation M3 pour l'établissement des courbes. Les tubes sont restés une nuit à 37° et 48 heures à 0° C.

La figure 6 montre la courbe de précipitation quantitative de la fraction M3–P.D. Le précipité est moins soluble dans un excès d'anticorps que les précipités obtenus avec la fraction M2–P.D.

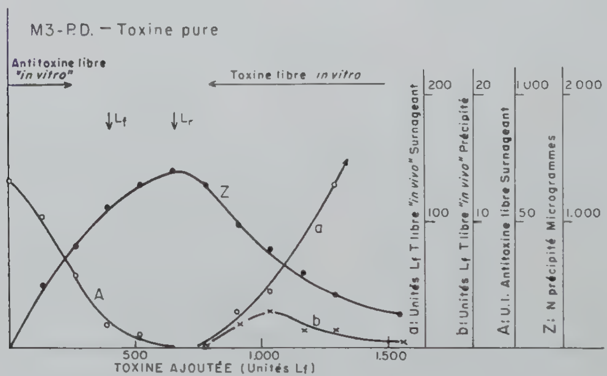

Fig. 6.

Les courbes des mélanges artificiels ayant une valeur Lr/Lf = 2,77 et 3,90 (fig. 7), sont plus longues que celles obtenues avec les mélanges des fractions M2. Il n'y a plus de formation de précipité après séjour à 0° C. On remarque de nouveau qu'on ne dépasse pas une certaine valeur limite d'azote précipitable. On retrouve de la toxine libre "in vitro" avant le point Lr dans les deux cas pour 800 unités Lf de toxine. Il y a donc de nouveau dans ces surnageants des complexes "toxine-antitoxine" solubles et non toxiques qui peuvent réagir avec des anticorps précipitants comme on l'a déjà remarqué dans l'étude des anticorps neutralisants non précipitants.

On peut aussi se demander si les anticorps non précipitants ne sont pas des anticorps mal adaptés au motif toxique et si ce n'est pas pour cette raison qu'il n'y a pas de précipitation.

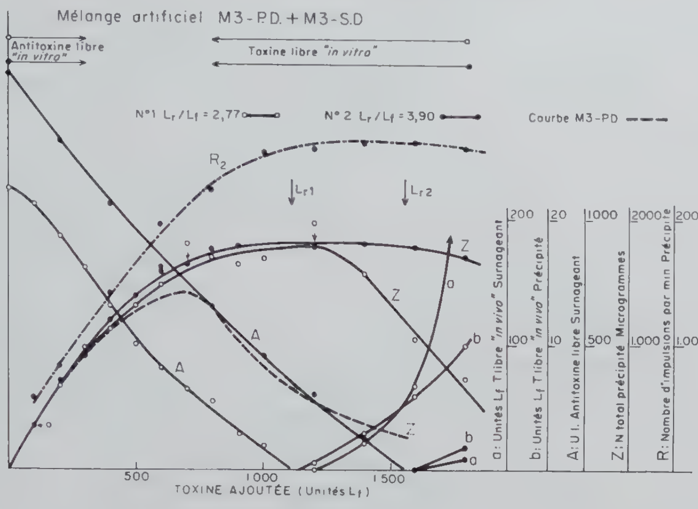

Fig. 7.

Un grand excès d'anticorps non précipitants devrait, dans ce cas, inhiber la réaction avec des anticorps précipitants.

L'expérience suivante permet de rejeter cette explication. La préparation M3–S.D. mélangée à volume égal avec le sérum M3 brut, donne un mélange de rapport Lr/Lf égal à 32,9. Nous avons d'abord ajouté des quantités variables de toxine à 0,5 ml de M3–S.D. Le volume final égale 0,8 ml. Après un séjour d'une heure à 37° C., suivi de l'addition de 0,5 ml de sérum brut, un précipité se forme après peu de temps. La série témoin a été précipitée dans les mêmes conditions, en remplaçant le sérum digéré par du tampon.

On trouve les deux courbes de précipitation quantitative déterminée après une nuit à 37° C. et 2 jours à 0° C., dans la figure 8. La quantité de précipité obtenue avec le mélange est plus élevée qu'avec le sérum brut. Les anticorps non précipitants n'inhibent donc pas la réaction avec le sérum précipitant et cette expérience plaide à nouveau en faveur d'une réaction entre une molécule présentant plusieurs motifs antigéniques et des anticorps distincts dirigés contre chacun de ces motifs.

Les courbes de précipitation quantitative des sérums de chevaux à faible pouvoir neutralisant ont déjà fait l'objet d'une étude détaillée (Relyveld et Raynaud, 1959c). Des anticorps neutralisants ont été ajoutés à la fraction 825 beta-2 ayant une valeur Lr/Lf = 0,23 de façon à obtenir une préparation mixte (sérum de

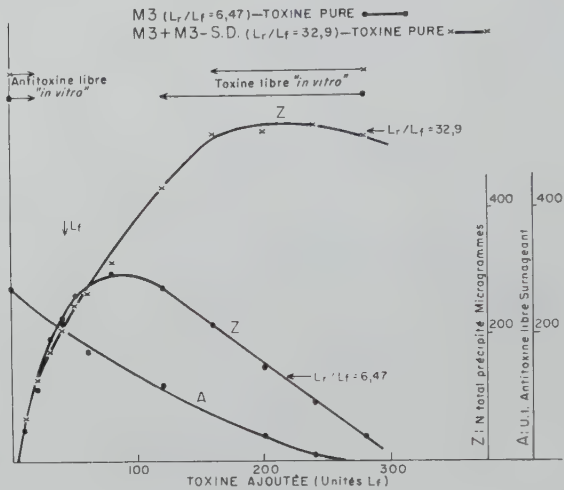

Fig. 8.

cheval 825 + sérum de mouton) présentant un rapport Lr/Lf = 1.

La figure 9 montre l'augmentation de l'azote précipitable. Le précipité de cette fraction du sérum 825 est toxique tout au long de la courbe; les précipités obtenus avec la préparation mixte ne deviennent toxiques qu'àprès le point Lr.

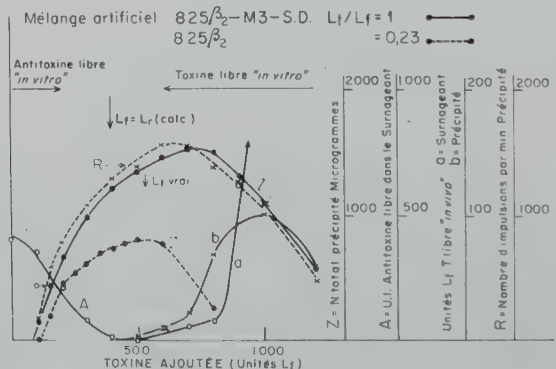

Fig. 9.

La technique de marquage des protéines à l'iode 131, telle que nous l'avons mise au point pour l'obtention d'une toxine radioactive, a été employée pour marquer les anticorps non précipitants.

La radioactivité de la préparation contenant 550 U.I/ml était de 58.760 i.p.m. par ml de solution. La quantité d'antitoxine spéci-

fiquement marquée était néanmoins très faible, d'environ 5%. Nous donnons, à titre indicatif, les courbes de radioactivité des précipités obtenus dans deux études quantitatives où les préparations d'anticorps non précipitants radioactifs ont été mélangés avec des anticorps précipitants radioactifs.

La radioactivité (figures 7 et 9) est proportionnelle à la quantité d'azote précipité.

Le tableau 4 montre qu'il n'y a pas d'entraînement de la toxine diphtérique en présence d'un autre système floculant, dans cet exemple, celui de la toxine et de l'antitoxine tétanique. Ces résultats sont identiques à ceux déjà obtenus par Pappenheimer et Yoneda (1957) et montrent qu'il n'y a pas de précipitation de la toxine par entraînement dans un précipité hétérologue.

TABLEAU 4.

Tube	Sérum antitétanique	Tox. dipht.	Tox. tét.	Tampon	% de radioactivité ds le précipité	
					précipité	surnageant
A	1 ml	0,5 ml	0,5 ml	3 ml	0,83	95
B	1 ml	0,5 ml	1,0 ml	2,5 ml	2,08	98,6
C	1 ml	0,5 ml	1,5 ml	2 ml	1,2	102,5

Toxine diphtérique marquée à l'iode 131 500 UF/ml
Toxine tétanique 400 UF/ml
Sérum antitétanique 400 UF/ml

Les coefficients numériques que l'on peut déterminer à partir des courbes de précipitation sont rapportées dans le tableau 5.

3) Etude de la mobilité électrophorètique des anticorps non précipitants par électrophorèse sur colonne.

Les anticorps précipitants de mouton ont une mobilité électrophorètique intermédiaire entre celles des beta-2-globulines et des gamma-globulines de cheval.

Nous les avons mise en évidence par immuno-électrophorèse et électrophorèse en gélose.

La fraction sérique portant ces anticorps a été nommée fraction gamma–S. Nous avons voulu vérifier si les anticorps non précipitants de mouton ont la même mobilité électrophorètique que les anticorps précipitants. Rien ne laisse prévoir si la ligne observée en immuno–électrophorèse est bien celle des deux anticorps réagissant avec la

TABLEAU 5.

Propriétés numériques des sérums étudiés.

Courbe	Lr/Lf	xm/xf	yf	ym	ym/yf	A/T(Lf)	A/T(M)	Figure
M2–Toxine	2,95	2,25	3,98	4,38	1,10	5,22	2,55	1
M2–Antitoxine	2,95	2,25	3,75	4,55	1,21	4,94	2,64	1
M2–P.D.	1,91	1,56	2,66	3,12	1,17	5,94	4,45	2
M2–P.D. + S.D.	2,89	2,0	3,03	3,81	1,26	6,76	4,25	5
M2–P.D. + S.D.	3,90	2,0	3,03	3,88	1,28	6,76	4,33	5
M3–P.D.	1,65	1,95	2,43	2,59	1,07	5,42	2,97	6
M3–P.D. + S.D.	2,77	3,0	2,45	3,53	1,44	5,48	2,63	7
M3–P.D. + S.D.	3,90	3,0	2,63	3,53	1,34	5,86	2,63	7
825–β_2	0,23	1,25	1,52	1,59	1,04	2,10	2,00	9
825–β_2 + M3–S.D.*	1,00	1,87	2,66	3,20	1,20	***	***	9
825–β_2+M3–S.D.**	0,77	1,44	2,29	2,46	1,07	***	***	–
M3	6,47	1,88	5,30	6,40	1,21	6,98	4,48	8
M3 + M3–S.D.	32,9	5,65	4,86	10,75	2,22	***	***	8

xm/xf = rapport des abscisses des points M (maximum de précipitation) et Lf (point correspondant à la floculation initiale).

yf = N anticorps précipité au point Lf (pour une unité de floculation de sérum), en microgrammes.

ym = N anticorps précipité au point M (pour une unité de floculation de sérum), en microgrammes.

A/T (Lf) = rapport moléculaire anticorps/toxine dans le précipité spécifique recueilli au point Lf (floculation initiale).

A/T (M) = rapport moléculaire anticorps/toxine dans le précipité spécifique recueilli au point M.

* = valeurs calculées

** = valeurs réelles (voir figure 16).

*** = les valeurs A/T aux points Lf et M n'ont pas été calculées dans ces cas, car les poids moléculaires des anticorps (intacts et digérés) ne sont pas identiques.

toxine, ou uniquement celle formée avec les anticorps précipitants.

Les anticorps précipitants et neutralisants ont été mis en évidence après électrophorèse sur une colonne de cellulose de type Porath (Porath 1956, 1957a, 1957b). Une description détaillée de l'appareil que nous avons construit et de la technique employée sera donnée ailleurs (Relyveld et Raynaud 1961).

L'électrophorèse du sérum de cheval sur colonne de cellulose a déjà été faite en 1957 par Gédin et Porath, qui retrouvèrent la plupart des anticorps floculants dans la fraction beta-2-globulines et une petite quantité dans la fraction gamma-globulines.

La mobilité électrophorètique des anticorps neutralisants de

cheval a été déterminée sur papier par SCHULTZE (1954) qui les a retrouvés dans les fractions beta-2 et gamma-globulines.

Les anticorps neutralisants et précipitants ont donc, dans ces derniers cas, la même mobilité.

Ces résultats concordent avec ceux d'autres auteurs qui ont déterminé la mobilité des anticorps par électrophorèse libre du sérum total et de ses fractions, ou du sérum épuisé, par l'antigène spécifique (DEUTSCH et NICHOL 1948; KECKWICK 1941, KECKWICK et RECORD 1941, VAN DER SCHEER *et al.*, 1940a et b, 1941, TISELIUS et KABAT 1939).

Nous avons longuement dialysé le sérum de mouton brut contre le tampon d'électrophorèse (véronal $T/2 = 0{,}02$; pH $= 8{,}2$). La colonne de cellulose a une hauteur de 124 cm. L'électrophorèse de 10 ml de sérum dilué au 1/2 a été faite pendant 15 heures sous une tension de 2100 volts, le courant étant de 60 mA.

La densité optique des fractions recueillies dans un collecteur sous volume de 10 ml, a été déterminée à 275 mμ. L'électroprotéinogramme est représenté dans la figure 10. La présence d'anticorps précipitants a été dépistée par la technique de diffusion en gélose, telle qu'elle a été mise au point pour l'examen des surnageants des courbes de précipitation quantitative.

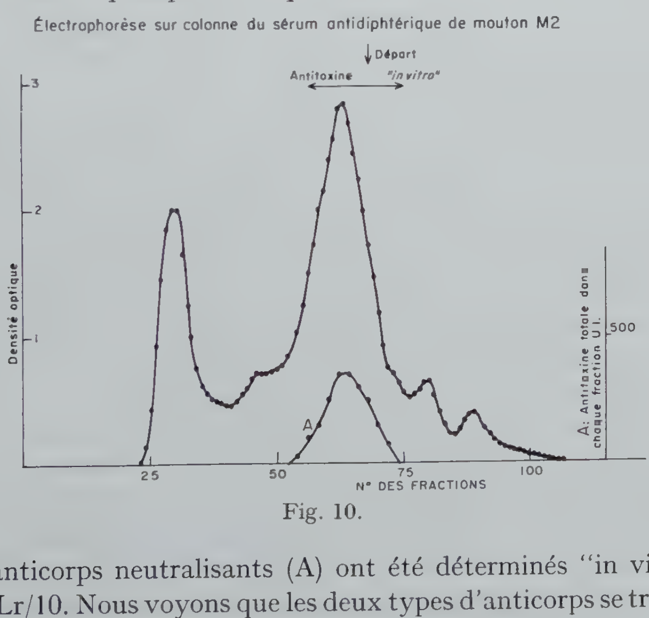

Fig. 10.

Les anticorps neutralisants (A) ont été déterminés "in vivo" au niveau Lr/10. Nous voyons que les deux types d'anticorps se trouvent dans la fraction gamma–S.

4) Electrophorèse et immuno-électrophorèse en gélose.

L'immuno-électrophorèse de certaines des préparations d'antitoxine étudiées est représentée en fig. 11. Cette image est l'inverse de celle que nous avons présentée dans une autre étude, où nous avons fait l'électrophorèse de la toxine brute et pure avant et après épuisement par antitoxine (Relyveld *et al.*, 1956).

Nous voyons de nouveau disparaître la ligne correspondant au système toxine-antitoxine. Le précipité spécifique obtenu à l'optimum de floculation et digéré ne contient pas d'anticorps accessoires comme le montre la détection de la fraction M3–P.D. avec une toxine brute et pure.

La présence de ces anticorps dans le précipité dépend naturellement de leur concentration dans le sérum brut ainsi que de celle des antigènes correspondants dans la préparation d'anatoxine employée pour la précipitation. La préparation M2–P.D. contenait, par exemple, à côté des anticorps antitoxiques, également des anticorps accessoires.

L'électrophorèse en gélose des fractions de M3 fig. 12 (p. 366) montre la diminution de la fraction gamma–S après épuisement des anticorps précipitants. Les fractions digérées ont la même mobilité.

Nous avons étudié la mobilité électrophorètique d'une toxine pure à laquelle ont été ajouté des quantités variables (50,100 et 200%) d'antitoxine neutralisante non précipitante (M3–S.D.).

La toxine a, dans les conditions de l'immuno-électrophorèse, une mobilité de 5.10^{-5} cm^2. $volt^{-1}$. $sec.^{-1}$ (Relyveld *et al.*, 1956).

La réaction des anticorps digérés avec la toxine entraîne la formation de complexes solubles qui ont, comme le montre la fig. 13 (p. 368) une mobilité comprise entre celle de la toxine et celle de l'antitoxine digérée, et légèrement supérieure à la mobilité des beta-2-globulines de cheval. Ces complexes ont été mis en évidence grâce à des anticorps précipitants. L'électrophorèse a été faite après un séjour des mélanges à 37° C. pendant une nuit.

La mobilité des anticorps neutralisants est représentée par la fraction M3–P.D. Cette fraction donne aussi avec la toxine et l'antitoxine de cheval diffusant à partir des rigoles latérales, la réaction d'identité récemment décrite par Osserman (1960). Les anticorps précipitants contenus dans le sérum de cheval 825 et les anticorps précipitants contenus dans la fraction M3–P.D. réagissent avec la toxine diphtérique en donnant une réaction d'identité.

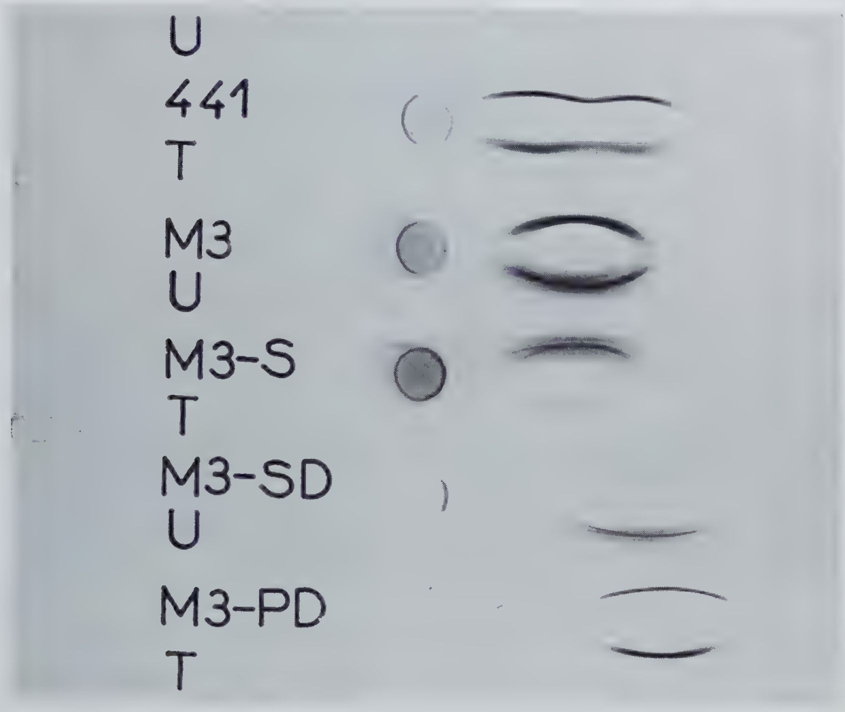

Fig. 11.

Immuno-électrophorèse des préparations obtenues à partir du sérum antidiphtérique de mouton M3. La mobilité des anticorps de mouton γ–S et γ'–S (anticorps digérés) a été comparée à celle des anticorps beta–2 et gamma de cheval présents dans le sérum 441 (Relyveld, 1959b).

441	: Sérum de cheval N° 441 dilué au 1/2,25 0,1 ml.
M3	: Sérum de mouton N° M3 brut, dilué au 1/2 0,15 ml.
M3–S	: Surnageant obtenu à l'optimum de floculation du sérum M3, dilué au 1/2 0,15 ml.
M3–S.D.	: Surnageant obtenu à l'optimum de floculation du sérum M3 digéré et purifié, dilué au 1/5 0,10 ml.
M3–P.D.	: Précipité spécifique toxine-antitoxine obtenu à l'optimum de floculation de la préparation M3, digéré et purifié, dilué au 1/5 0,1 ml.

Les caractères des préparations sont présentés dans le tableau 3. Dans les rigoles latérales : U = toxine diphtérique brute ultrafiltrée à 100 UF/ml. T = toxine pure à 100 UF/ml. Chaque fois 1,5 ml.
Durée de l'électrophorèse : 4 heures.
Tension mesurée aux bords des plaques (4) : 70 volts.
Intensité totale : 220 mA.

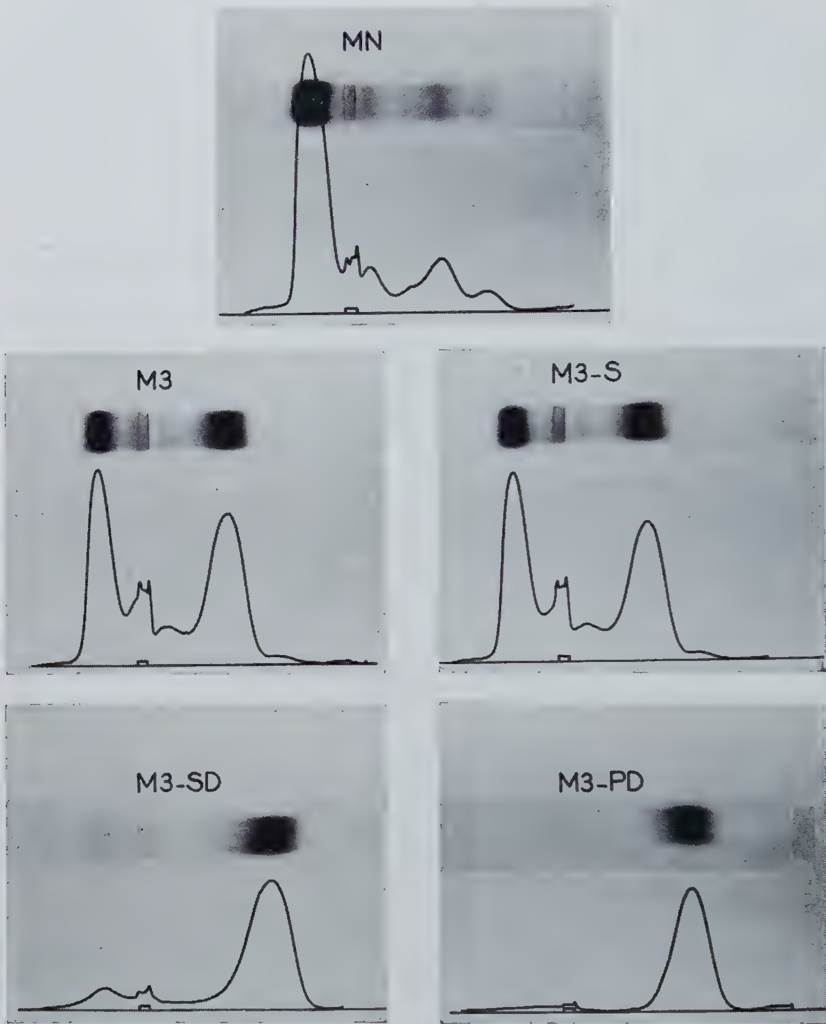

Fig. 12.

La préparation contenant la plus faible quantité d'antitoxine non précipitante (50%), contient, à côté des complexes solubles, encore de la toxine libre; les deux constituants donnent une réaction d'identité.

D'après les résultats de l'électrophorèse, les complexes fournis par la toxine et les anticorps non précipitants ont tous la même vitesse électrophorètique.

5) Etude des fractions par la technique de précipitation spécifique en gélose.

Les fractions du sérum M3 sont présentées en double diffusion sur plaque dans la fig. 14 (p. 370). Nous voyons qu'on peut encore détecter des traces d'anticorps précipitants dans les fractions M3–S et M3–S.D. pour les concentrations élevées de ces préparations. Le fait que les surnageants des courbes de précipitation quantitative "in vitro" donnent une réaction négative, indique donc seulement que les anticorps précipitants résiduels y sont contenus à une concentration inférieure à 2,5 UF/ml (limite de la réaction dans ces conditions).

La précipitation en gélose nous a fourni des indications supplémentaires sur l'intéraction des anticorps précipitants et neutralisants avec la toxine.

Des anticorps non précipitants marquées à l'iode 131 ont illustré la nature de la réaction. Nous avons fait diffuser contre la toxine pure la fraction beta-2 du sérum 825 (rapport in vivo/in vitro = 0,23) et le mélange obtenu à partir de ce serum et des anticorps non précipitants marquées à l'iode 131, de façon à ce que le rapport in vivo/in vitro = 1. La réaction de précipitation spécifique sur plaque (fig. 15, p. 372) montre dans les deux cas la formation d'une seule ligne. Mais l'immunoradiogramme montre que seule celle formée à partir du mélange est radioactive et que les anticorps non précipitants radioactifs sont fixés

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Fig. 12.

Electrophorèse en gélose, détermination des courbes d'extinction après coloration par l'amidoschwarz.

Les préparations suivantes ont été étudiées:

MN : Sérum de mouton normal dilué au 1/10 0,15 ml.
M3 : Sérum antidiphtérique de mouton N° M3 dilué au 1/10 0,15 ml.
M3–S : Surnageant obtenu à l'optimum de floculation de la préparation M3, dilué au 1/10 0,165 ml.
M3–S.D. : Surnageant obtenu à l'optimum de floculation de la préparation M3 digéré et purifié, dilué au 1/40 0,15 ml.
M3-P.D. : Précipité spécifique toxine-antitoxine diphtérique obtenu à l'optimum de floculation de la préparation M3 digéré et purifié, dilué au 1/4 0,15 ml.

Les caractères des préparations sont présentés dans le tableau 3.

Les conditions de l'électrophorèses sont celles décrites dans la légende de la figure 11.

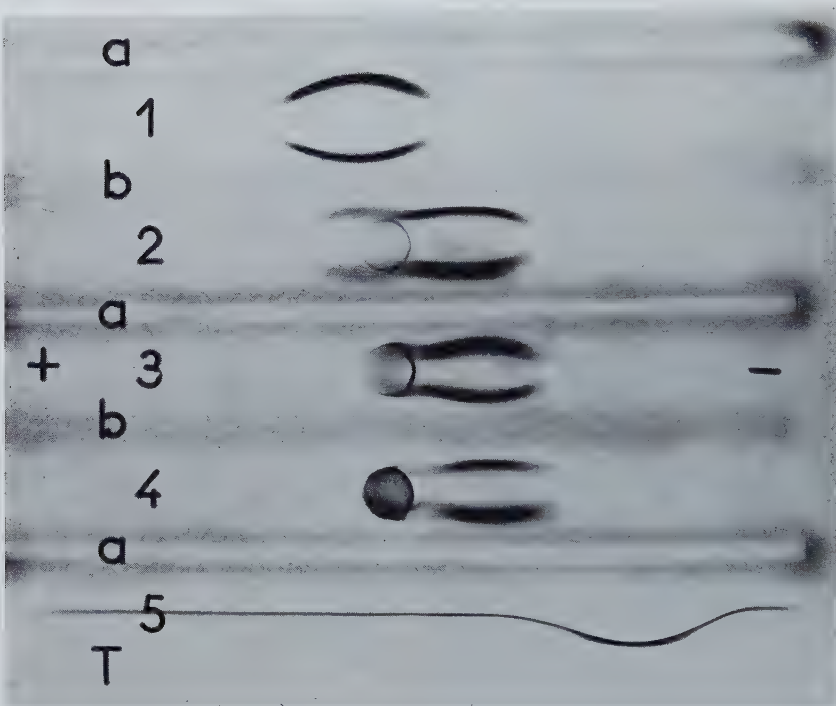

Fig. 13.

Immuno-électrophorèse de la toxine diphtérique neutralisée par des anticorps non précipitants.

Divers mélanges ont été étudiés:

1 : Toxine pure à 200 UF/ml
2 : Toxine pure à 200 UF/ml + anticorps non précipitants 100 U.I/ml (M3-S.D.)
3 : Même mélange mais avec 200 U.I. d'anticorps non précipitants par ml.
4 : Même mélange mais avec 400 U.I. d'anticorps non précipitants par ml.
5 : Fraction M3-P.D. à 100 UF/ml. Dans chaque réservoir 0,1 ml.

Dans les rigoles : a = 825 beta-2 à 200 UF/ml
b = la même préparation à 100 UF/ml
T = toxine pure à 100 UF/ml.

Chaque fois 1,5 ml.

Les conditions de l'électrophorèse sont celles décrites dans la légende de la figure 11.

sur la ligne formée avec les anticorps précipitants.

Il n'y a pas de radioactivité détectable si on laisse diffuser la fraction non précipitable radioactive contre la toxine. Les plaques ont été photographiées après 9 jours à 37° C. et 5 jours de lavage à l'eau physiologique. Le liquide de lavage (5 litres) a été renouvelé deux fois par jour. Les résultats de cette éxpérience sont de nouveau en faveur de l'interprétation de la réaction "toxine-antitoxine diphtérique", comme étant celle entre une molécule de toxine et des anticorps précipitants et non précipitants dirigés contre des motifs antigéniques distincts.

6) Influence des anticorps non précipitants sur l'optimum et la vitesse de floculation.

Il nous a paru intéressant d'étudier l'influence des anticorps non précipitants sur la vitesse et l'optimum de floculation.

On sait qu'on peut observer des différences de titres suivant les techniques employées (alpha ou beta), mais que ces différences disparaissent si l'on opère dans les conditions définies par Bowen et Wyman (1953a et b) selon une technique que nous avons proposé d'appeler technique gamma (Raynaud et Relyveld 1959).

Nous avons étudié l'influence des anticorps non précipitants de mouton (préparation M3-S.D. : 2250 Lr/ml) sur 2 systèmes floculants: sérum de cheval 825-beta-2 Lr/Lf = 0,23 et sérum de mouton précipitant M3–P.D. Lr/Lf = 1,65.

Nous avons employé la technique Ramon à volume variable dans les conditions suivantes:

Toxine purifiée 80 Lf/ml 1 ml – Solution d'anticorps 0,1 à 1 ml.

Les tubes ont été laissés à 45° jusqu' à apparition de la floculation dans un des tubes et ramenés à partir de ce moment à la température ordinaire. La floculation dans les tubes voisins est ainsi artificiellement retardée, ce qui permet une observation plus facile de l'optimum.

La solution d'anticorps étant obtenue en mélangeant à un volume de 825-beta-2 (350 Lf/ml) des volumes variables de la préparation de M3–S.D. (2250 Lr/ml) et en complétant à 2 volumes.

La composition des mélanges est rapportée dans le tableau 6. D'après le nombre d'unités Lf 825 et d'unités antitoxiques (825 beta-2 et M3–S.D.) employées, on a calculé le rapport Lr/Lf.

Nous avons vérifié que le titre neutralisant du mélange est égal à

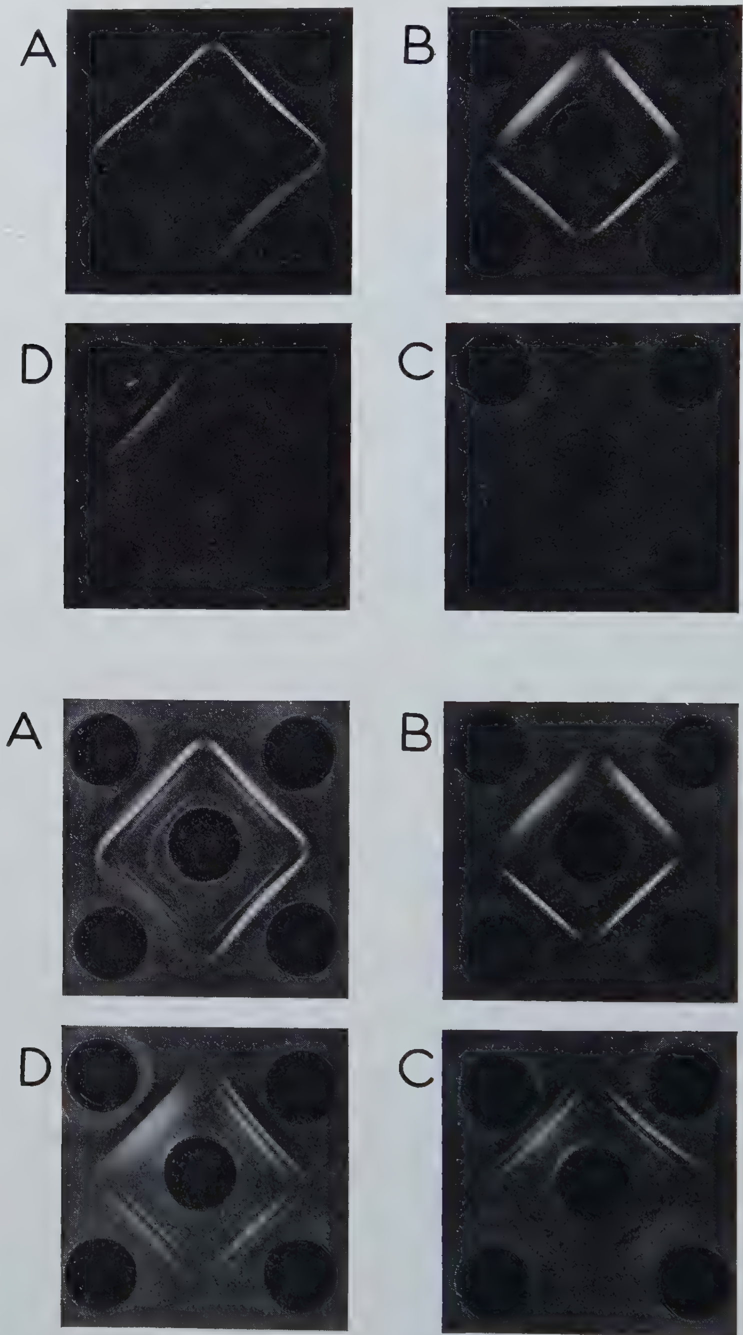

Fig. 14.

la somme des unités ajoutées, qu'elles proviennent du sérum 825 ou du sérum M3–S.D.

TABLEAU 6.

Influence des anticorps non précipitants (M3-S.D.) sur le titre floculant et le Kf de la préparation 825–beta–2.

UF/ml (825)	Lr/ml (825 + M3 –S.D.)	Rapports Lr/Lf calculés	Valeurs trouvées		
			Lf/ml	Lr/Lf	Kf
175	40	0,23	175	0,23	1 h 20′
175	96,2	0,55	189	0,509	15′
175	199,5	1,14	250	0,798	7,5′
175	288,5	1,65	267	1,08	6′
175	407	2,33	320	1,27	9′
175	645	3,69	320	2,02	16′
175	882	5,04	300	2,94	23′
175	1165	6,66	267	4,36	31′

Par contre, la détermination expérimentale du titre floculant, montre que ce dernier augmente en fonction de la teneur en anticorps coprécipitants. Cette augmentation n'est pas indéfinie, et on obtient une valeur plafond, voisine du double de la valeur initiale. Ce maximum est atteint sensiblement à partir du moment où le mélange présente un rapport Lr/Lf = 2,33.

Pour les valeurs supérieures, le titre floculant montre une légère baisse, il reste à déterminer si l'écart observé est vraiment significatif.

Le Kf subit une diminution considérable en passant par un

Fig. 14.

Mise en évidence du nombre de constituants présents dans les fractions de M3, par la technique de précipitation spécifique sur plaque.

La toxine se trouve au centre; en haut les fractions testées contre une toxine pure à 150 UF/ml; en bas contre une toxine brute ultrafiltrée également à 150 UF/ml. Les fractions ont été testées à diverses dilutions en commençant en haut à gauche et en suivant le sens des aiguilles d'une montre.

A = M3: non dilué, 1/2, 1/4 et 1/8.

B = M3-P.D.: non dilué, 1/2, 1/4 et 1/8.

C = M3-S.: non dilué, 1/3, 1/6 et 1/12.

D = M3-S.D.: non dilué, 1/5, 1/10 et 1/20.

Dans chaque réservoir 0,5 ml.

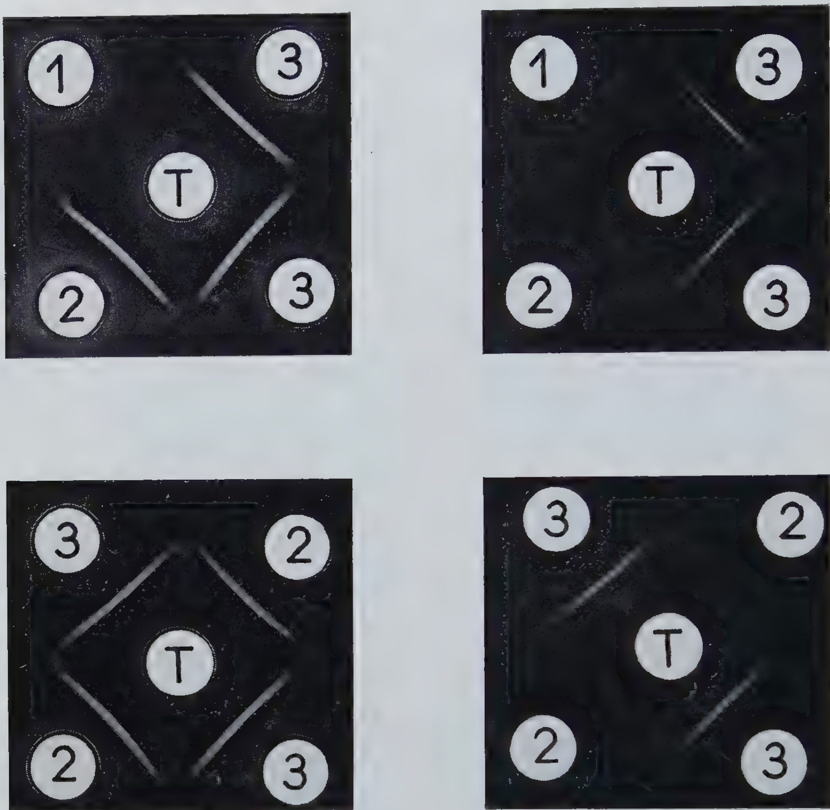

Fig. 15.

1 : M3-S.D. marqué à l'iode 131, concentration 200 Lr/ml.
2 : 825 fraction beta-2 à 200 UF/ml, Lr/Lf = 0,23.
3 : Mélange 825–beta–2 et M3-S.D. radioactif de façon que le rapport Lr/Lf soit égal à 1, 200 UF/ml.
T : Toxine pure à 200 UF/ml.

Dans chaque réservoir, 0,5 ml. Distance entre les bords des réservoirs (diamètre 0,8 cm) : 1,5 cm.

Images de gauche : photographies directes des plaques.
Images de droite : radiogrammes.

minimum qui est atteint lorsque le rapport Lr/Lf est voisin de 1 (fig. 16, p. 373).

L'augmentation du titre n'est pas une augmentation apparente, dûe à l'intervention de la modification de la concentration totale (A + T). Nous l'avons vérifié, en effectuant la détermination du titre et du temps de floculation, suivant la technique gamma, dans

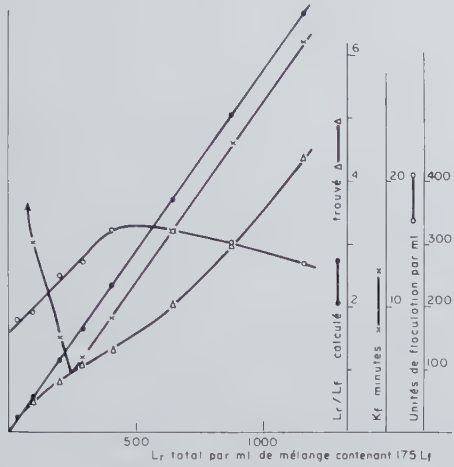

Fig. 16.

Influence des anticorps non précipitants (M3-S.D.) sur le titre floculant et le Kf de la préparation 825-beta-2 (Lr/Lf = 0,23).

le cas du mélange de rapport Lr/Lf = 2,33. Pour cela, nous avons admis qu'une U.I. de sérum M3–S.D. correspondant à 1 μg d'N[1]).

Nous avons alors déterminé le temps de floculation de ce mélange dans les conditions suivantes:

Toxine pure: solution à 800 Lf/ml = 0,1 ml.

Solution d'anticorps: contenant 87,5 Lf de sérum 825 par ml et 183,5 U.I. (M3–S.D.) par ml = 0,1 à 1 ml.

On complète chaque fois le volume de façon à réaliser une concentration totale (A + T) égale à 285 μg N/ ml.

La courbe représentant le Kf des diverses mélanges (fig. 17) montre que le titre obtenu est très voisin du titre déterminé par la technique Ramon à volume variable.

La même expérience répétée avec le sérum de mouton précipitant M3–P.D. Lr/Lf = 1,65 nous a montré que l'addition des anticorps non précipitants provoque seulement une baisse très peu significative du titre floculant et un allongement du Kf (tableau 7, fig. 18). Ce résultat qui isolé serait très difficile à comprendre, s'interprète

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1) Cette estimation ne constitue malheureusement qu'une approximation; elle est basée sur les différences d'N total précipitable.

Nous pensons cependant pour des raisons qui seront développées ultérieurement, que le calcul réel du taux d'N anticorps correspondant à une U.I. d'une antitoxine donnée nécessite des recherches complémentaires.

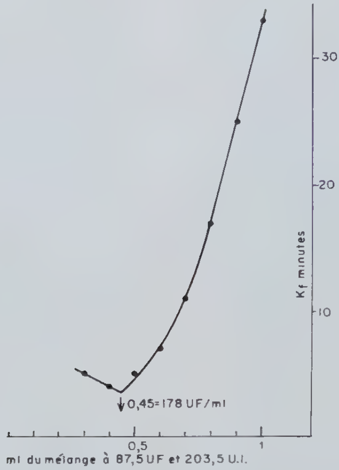

Fig. 17.

Détermination du titre floculant par la technique gamma. Le sérum est un mélange contenant 87,5 UF et 203,5 U.I. (volume variable). La toxine titre 800 UF/ml. Volume ajouté 0,1 ml.

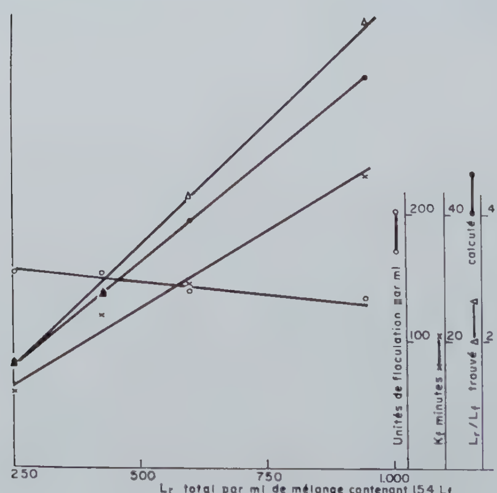

Fig. 18.

Influence des anticorps non précipitants (M3-S.D.) sur le titre floculant et le Kf de la préparation M3-P.D.

très facilement d'après les résultats précédents. Le mélange de départ ayant un rapport Lr/Lf de 1,65 a déjà atteint le "plateau" visible sur la figure 16.

TABLEAU 7.
Influence des anticorps non précipitants (M3–S.D.) sur le titre floculant et le Kf de la préparation M3–P.D.

UF/ml M3–P.D.	Lr/ml M3–P.D.+ M3–S.D.	rapports Lr/Lf calculés	Valeurs trouvées		
			Lf/ml	Lr/Lf	Kf
154	254	1,65	154	1,65	12′
154	427	2,77	154	2,77	24′
154	600	3,90	139	4,32	29′
154	947	6,15	133	7,12	46′

Ces phénomènes permettent de préciser la nature de la corrélation entre le titre floculant d'un sérum et la forme de la courbe de précipitation quantitative. Le point Lf est toujours à gauche du maximum de précipitation, mais l'écart entre ce point et le maximum est très variable d'un sérum à l'autre. Ces variations semblent sous la dépendance directe du taux relatif des anticorps coprécipitants et des divers anticorps précipitants.

Par ailleurs, les anticorps précipitants ne peuvent co-précipiter qu'une quantité limitée d'anticorps neutralisants non précipitants.

Lorsque le rapport L+/Lf est très faible, le sérum est capable de coprécipiter beaucoup plus d'anticorps coprécipitants artificiellement ajoutés. Les mélanges ainsi reconstitués permettent de retrouver les propriétés des divers sérums naturels.

Lorsque le rapport Lr/Lf est égal à 1, le Kf est à son minimum.

Pour les mélanges de rapport Lr/Lf $>$ 1, mais inférieur à une certaine valeur qui paraît voisine de 2, le sérum peut encore coprécipiter des quantités supplémentaires d'anticorps non précipitants. Mais le Kf augmente de même que l'N total précipitable.

A partir d'une certaine proportion des anticorps précipitants et non précipitants, correspondant sensiblement à un rapport Lr/Lf = 2 le mélange devient incapable de précipiter des quantités supplémentaires importantes d'anticorps coprécipitants. Le Kf cependant augmente. Le point Lr se trouve alors après le maximum de la courbe. Il reste à déterminer si la légère baisse de titre floculant noté dans la figure 16 est significative.

Discussion.

Les résultats rapportés ci-dessus établissent que l'on peut par une seule précipitation partielle effectuée avec un rapport antigène/anticorps correspondant au mélange qui donne la floculation initiale, éliminer d'un sérum de mouton présentant un rapport Lr/Lf élevé, tous les anticorps précipitants la toxine diphtérique. Le surnageant contient alors à titre élevé des anticorps neutralisants non précipitants.

Heidelberger et Kendall (1935) ont obtenu des résultats analogues en effectuant l'addition d'antigène par petites fractions successives, pour le système ovalbumine-antiovalbumine.

Les anticorps non précipitants neutralisants sont coprécipitables par des anticorps précipitants antidiphtériques de même espèce (mouton) ou d'espèce différente (cheval).

Si les anticorps précipitants sont non neutralisants (cheval 825), le mélange obtenu se comporte alors (pour certaines proportions des 2 constituants) comme un système précipitants neutralisant.

Un fait très important doit être souligné. La forme de la courbe de neutralisation (quantité d'anticorps neutralisants persistant dans un mélange Sérum + Toxine en fonction de la quantité de toxine ajoutée) est pratiquement rectiligne lorsqu'on a affaire à des anticorps neutralisants non précipitants "isolés" c'est à dire non accompagnés d'anticorps précipitants (figures 3 et 4).

Lorsque la même quantité d'anticorps neutralisants est mélangée à des anticorps précipitants, la courbe de neutralisation a la forme classique à concavité dirigée vers le haut (figures 1, 2, 5, 6, 7, 8, 9).

En l'absence d'anticorps précipitants non neutralisants, la neutralisation s'effectue donc selon des lois simplifiées, voisines d'une réaction stoechiométrique: une quantité donnée de sérum neutralise toujours la même quantité de toxine, quelle que soit la proportion relative des 2 réactifs dans le mélange.

Cette observation, qui demande à être développée et étendue, repose tout le problème des lois de combinaison entre la toxine et l'anticorps neutralisant correspondant.

Les particularités habituellement reconnues à cette combinaison résulteraient de l'hétérogénéité des anticorps présents dans tous les sérums antidiphtériques. Les anticorps précipitants non neutralisants modifieraient l'évolution de la réaction entre les anticorps neutralisants et la toxine, empêchant celle-ci de se manifester sous sa forme la plus simple.

Réciproquement, les anticorps non précipitants interviennent dans la floculation. En s'ajoutant au précipité spécifique, ils exercent une influence qui se manifeste par un changement de la vitesse de floculation et une modification de la valeur du rapport Antigène/Anticorps donnant la floculation initiale. Le facteur déterminant est alors le rapport entre ces 2 types d'anticorps. Grâce a leur rapport Lr/Lf élevé, c'est à dire grâce à leur teneur élevée en anticorps neutralisants (et non précipitants), les sérums de mouton hyperimmunisés permettent une élimination facile des anticorps précipitants. Une partie importante des anticorps neutralisants non précipitants est bien éliminée par coprécipitation, mais les taux résiduels dans le surnageant, des anticorps neutralisants non précipitants, sont si élevés que leur étude a pû être faite sans difficulté majeure.

Dans le cas des sérums de chevaux, le taux des anticorps "neutralisants" non précipitants est en général tel que par précipitation avec l'antigène intact, ils sont presque toujours coprécipités. Les anticorps neutralisant la toxine diphtérique sont donc certainement des anticorps non précipitants chez le mouton en général et chez certains chevaux, dans des cas exceptionnels. Ces sérums sont caractérisés par leur rapport Lr/Lf très élevé (supérieur à 2).

On sait depuis Barr et Glenny (1938) qu'il arrive parfois que chez certains chevaux, on peut avoir des anticorps non précipitants à titre élevé, mais il a été très difficile jusqu' à présent d'obtenir, à volonté ce type de réponse.

Pope et Stevens (1953) ont décrit une préparation d'antitoxine avec un rapport "in vivo/in vitro" égal à 1,55 ne donnant pas de précipité au point Lr.

Les anticorps neutralisants présents dans le sérum de la plupart des chevaux de rapport L+/Lf voisin de 1, sont donc aussi très probablement des anticorps non précipitants. Mais ils ne peuvent être préparés par absorption partielle du sérum par l'antigène intact. Il reste donc à trouver une méthode simple permettant de les isoler par absorption du sérum total par des fragments immunologiquement bien définis obtenus à partir de l'antigène.

Résumé.

La préparation d'anticorps diphtériques neutralisants non précipitants à partir de sérums de mouton avec un rapport in vivo/in vitro élevé a été décrite. L'étude quantitative de la réaction

toxine-antitoxine faite avec ces anticorps a montré que la neutralisation de l'antitoxine se fait suivant une règle simple. Il n'y a pas de formation de précipité, mais les anticorps peuvent être coprécipités par un système floculant.

La mobilité électrophorètique des anticorps a été étudiée par l'électrophorèse en gélose et par l'immuno-électrophorèse. Les complexes "toxine-antitoxine non précipitant" ont une mobilité voisine de celle des beta-2-globulines de cheval. L'électrophorèse sur colonne de cellulose a montré que les anticorps précipitants et les anticorps neutralisants non précipitants ont la même mobilité électrophorètique.

L'étude par double diffusion en gélose montre que les anticorps non précipitants radioactifs se fixent sur la ligne formée entre, d'une part, la toxine et d'autre part, les anticorps précipitants.

Le titre floculant des sérums de rapport Lr/Lf faible, augmente si l'on y ajoute des anticorps neutralisants non précipitants, le Kf des préparations baisse d'abord pour remonter ensuite en fonction de la quantité d'anticorps non précipitants ajoutée.

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(Laboratory of the Municipal Medical and Health Service of Amsterdam).

A SIMPLIFIED FERMENTATION TUBE FOR THE EXAMINATION OF DRINKING-WATER

by

TH. G. N. DRESSCHER

(Received April 14, 1960).

For the examination of the drinking-water supply of airports, seaports, aeroplanes and ships, it is sometimes necessary to gain some insight into the quality of the drinking-water on the spot.

Generally, however, bacteriological inspection of water supplies aboard or at ports is impossible because of the difficulties involved in carrying along the necessary equipment.

In particular, test-tubes, containing a liquid medium that will prove pollution of drinking-water, are difficult to carry because they must always be kept in a vertical position. When the tubes get into a horizontal position or when they are held upside down, the cotton plug in the mouth of the tube will be moistened or the fermentation tube (Durham tube), inserted in the test-tube, will lose a part or the whole of its contents.

These difficulties may be avoided if the tubes are prepared as follows:

Ordinary test-tubes are filled with either lactose-bile-litmus-peptone water, MacConkey's broth or some other medium for the presumptive coliform test. In each tube shall be $\pm$ 1 ml more liquid than is necessary for the examination of drinking-water.

Into the mouth of each tube a small but not too tight cotton plug is inserted and another one of normal size is used as a stopper (Fig.1).

After sterilization the stopper of each tube is separately taken off; the mouth of the tube is flamed and with the aid of a sterile pair of tweezers the small cotton plug is pushed so far down into the medium that there will be no bubble of air when the tube is held upside down (some skill is required). The stopper is then replaced (Fig. 2).

The tubes are now ready for transport and later use. It proved to be possible to carry and keep them in any position without loss of liquid.

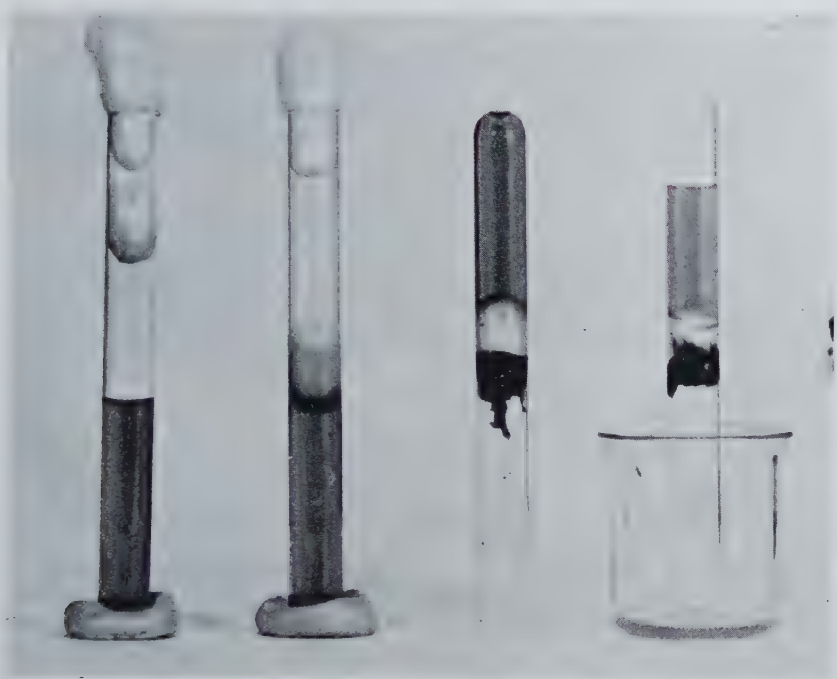

Fig. 1. Fig. 2. Fig. 3. Fig. 4.

For the detection of pollution of drinking-water, the tubes should be used as follows:

1. Take off the stopper and flame the mouth of the tube.
2. Remove the second moist cotton plug with a sterile pair of tweezers and discard it.
3. Allow the desired quantity of drinking-water to run into the tube out of the water-tap, which has been flamed already in the beginning of the procedure.
4. Replace the stopper lightly.
5. Mix water and medium by tapping the tube on the hand.
6. Flame the stopper.
7. Press the flamed stopper into the liquid with the aid of a sterile pair of tweezers. This again should be done in such a way that there

will be no bubble of air when the tube is held upside down. Fig. 3 shows a slight failure: bubble of air.

8. Put the tube upside down in a beaker. The beaker should be put in a warm place, for example 35° C. and left for 24 hours at least.

If the water is polluted, the liquid will start fermenting and there will be formation of gas. The gas will press the liquid through the cotton plug into the beaker in which the tube has been placed (Fig. 4).

Obviously the application of the described fermentation tube is not restricted to the examination of drinking-water; in view of its simplicity and negligible cost it may be a useful alternative to Einhorn and Durham tubes in other types of fermentation tests as well.

Summary.

A simplified fermentation tube has been described.

(Research Department, British Vinegars Ltd., Frome, Somerset, England, and Research Station, Long Ashton, Bristol, England).

ARE SPECIES OF BACTERIA UNCLASSIFIABLE?

by

J. L. SHIMWELL and **J. G. CARR**

(Received June 1, 1960).

INTRODUCTION.

In recent papers (SHIMWELL, 1956, 1957, 1959) it has been shown that 'mutation' is rampant in the genus *Acetobacter*. Nearly all strains of all available *Acetobacter* species examined contained and/or readily gave rise to a variety of 'mutant' colony forms which, on separate picking and cultivation, were often found to have lost or gained some major correlated characteristic which shifted them into another species and even into another of FRATEUR's (1950) groups. Such mutants could often also give rise to still further mutants which had similarly again changed their 'species'. It was, indeed, rare to find an *Acetobacter* culture in which all the separate colonies produced had the same properties as had the culture itself as a whole. The arch-example of this was one culture of *A. mesoxydans*, which gave three types of colony not one of which had the same properties as the parent culture when studied as a whole. This latter culture, indeed, could not be 'purified' by plating, as the picking of any individual colony resulted in a culture with properties different from those of the parent culture.

The different properties shown by different colony forms obtained from a particular culture of a named species often varied, not only biochemically, but often also in their cultural properties, cell-form, growth rate, and so on.

From the above it seemed impossible to escape the conclusion that 'species' of *Acetobacter* cannot be classified at all, no matter what criteria are used, for even in the very course of studying a culture, its combination of properties change, often with kaleidoscopic rapidity and effect.

As a result of this re-assessment of the genus *Acetobacter* the new edition of the catalogue of the National Collection of Industrial Bacteria (N.C.I.B.) – now in press – is prefacing the section on *Acetobacter* with a paragraph to the effect that the rampant mutation in this genus prevents any culture from being guaranteed either as to name or purity (private communication from the Curator).

In the course of extensive postal and verbal discussions of the papers mentioned above, most other workers have assumed that in this extraordinary facility of mutation, and in the high frequency of occurrence of mutants in almost all cultures, the genus *Acetobacter* is unique. Indeed, the late Dr. R. S. Breed, in a private communication to one of us (J. L. S.) wrote that it was fortunate that this was so, otherwise strains of other genera, in collections, would not have shown such constancy of properties and purity over such long periods. In general, this attitude was that of most correspondents.

However, one or two specialists, working on particular genera, wrote to say that they had found much the same frequency of mutants in cultures of their own particular specialized groups, and had reached the conclusion that their species, too, were unclassifiable. It also seemed to us, from first principles, that it would indeed be remarkable if the one genus we had happened to study extensively (primarily because of its industrial and economic significance) were to be, of all genera, t h e o n l y o n e to exhibit such extraordinarily high mutant frequency. It was recognised, of course, that 'variation' in bacteria had been recorded to some extent from the earliest times, and we were also aware that the occurrence of mutation has been recorded in a number of bacteria of different types. This, however, has apparently not been a matter of great concern to taxonomists, who have continued their classification studies on much the same lines as previously.

Since the publication of the papers mentioned above, we have encountered several instances of *Acetobacter* strains giving rise to mutants which have completely lost the power of producing acetic acid from ethanol, whilst retaining their other properties, therefore mutating right out of the genus *Acetobacter* altogether. Conversely, one of these 'quasi-acetobacters', with the rare power of producing voluminous extra-cellular starch, has given rise to mutants which correspond to two of the best known classical species of *Acetobacter*, namely *A. pasteurianus* and *A. rancens*, both of these having g a i n e d the power of producing acetic acid, and one of them having

simultaneously lost the power of producing starch. These quasi-acetobacters do not seem classifiable in any existing genus, and if they had been encountered in some natural habitat might never have been recognised either as the offspring or as the progenitors of acetic acid bacteria (SHIMWELL and CARR, 1960).

In view of the foregoing it was considered of interest to investigate the existence and frequency of occurrence of mutants in a relatively small, randomly selected (but representative) collection from randomly selected genera. Eleven strains from ten widely different genera were therefore taken at random. The only element of 'selection' was in choosing, as far as possible, genera with which we were relatively unfamiliar, and in obtaining them from widely different habitats and sources.

It was considered that the frequency of occurrence of 'mutants' in such a collection of randomly selected strains of different genera might reasonably be assumed to indicate what might be expected in other genera.

METHODS.

To obtain photographable colonies, serial dilutions of all cultures were smeared with a glass spreader on the surface of solidified agar plates of various compositions appropriate to the type of bacterium concerned. Plates of aerobic, anaerobic, and micro-aerophilic strains were respectively incubated under oxygen tensions appropriate to their requirements.

The resulting colonies were observed and photographed by transmitted or oblique transmitted light.

It soon became apparent that to determine whether all different colony forms, produced by the various strains, were correlated with different biochemical or other properties involved a specialist knowledge of the multifarious biochemical, nutritional, physiological, and other characteristics of each genus, and even of each randomly selected species; and this we did not possess. However, in some cases such correlated differences virtually exhibited themselves; where this occurred they have been recorded.

RESULTS.

The results obtained with the various strains studied are given in the order in which they were obtained, rather than alphabetically

according to the generic name, or as these occur in, *e.g.*, Bergey's Manual.

1. *Erwinia carotovora* (N.C.I.B. 8093). When this was plated on yeast-extract (YE)/glycerol/agar three colony forms were obtained, namely (1) large smooth circular opaque; (2) very small ditto and (3) translucent circular granulated. Fig. 1 shows these, and also a smooth opaque papilla arising from a translucent granulated colony, together with a granulated translucent colony arising from the periphery of a large circular opaque smooth one.

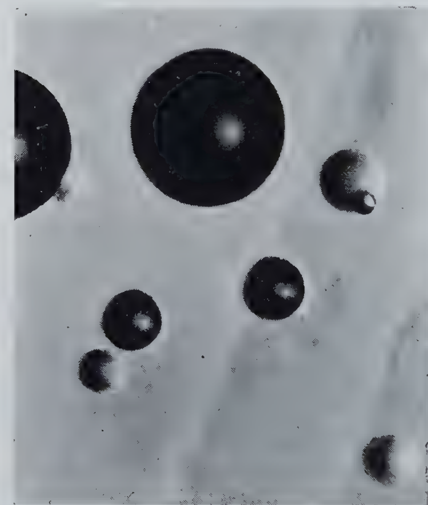

Fig. 1. Colonies of *Erwinia carotovora*. YE/glycerol/agar. × 40.

Fig. 2. Colonies of *Er. carotovora*. 'nutrient' agar. × 40.

When the same dilution was plated on 'nutrient' (peptone/meat-extract/agar), a very different picture was obtained, a l l colonies being irregularly circular, translucent, and relatively featureless (Fig. 2).

This rather suggests that the different genotypes present in the dilution could only express themselves as different phenotypes when supplied with either the growth factors in yeast-extract or with glycerol.

2. *Photobacterium* strain. (National Collection of Marine Bacteria). This highly luminous strain, when plated on 1% YE/2.3%NaCl/

agar, showed a great variety of colony forms of which 5 are visible on Figs 3 and 4 (same plate). Few colonies on the plate were devoid of papillae, outgrowths, or sectors. Indeed, the types of sectoring present included almost all those described in the literature of bacterial mutation, *i.e.* $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{2}$–twin, $\frac{1}{4}$, $\frac{1}{8}$, and so on. Fig. 4 shows at least one sector on the papillated colony.

Fig. 3. *Photobacterium* strain. Colonies on YE/NaCl/agar. × 40.

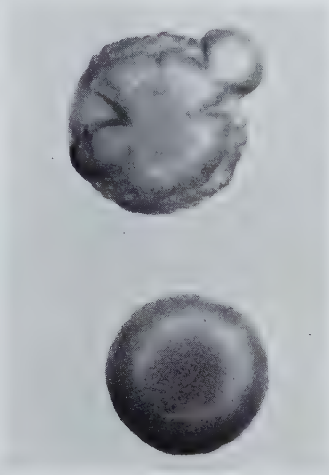

Fig. 4. *Photobacterium* strain. Colonies on YE/NaCl/agar. × 40.

Cultures derived by picking different colony forms, papillae, and sectors, however, were all luminous, although the degree of luminosity varied considerably; other characteristics were not investigated.

3. *Lactobacillus* sp. Heterofermentative, isolated from cider by one of us (J. G. C.). On apple–juice agar 3 colony forms were obtained (Fig. 5). When picking these, the large irregular colony illustrated was intensely viscid, drawing out into a long thread with the wire. The other two colony types were not ropy; nor did the culture as a whole produce ropiness in cider.

Cultures derived by separately picking the 3 colony forms illustrated were then replated on the same medium. Despite this 'purification' a similar mixture of colony forms was obtained again in each case.

4. *Lactobacillus* sp. Homofermentative; catalase positive, isolated from cider by one of us (J. G. C.). This strain, with its

unusual, but not unique, catalase positivity, together with its homofermentative nature, could virtually be regarded as of a different genus from the catalase-negative, heterofermentative strain as described above; but we have not so regarded it for the purpose of the present investigation.

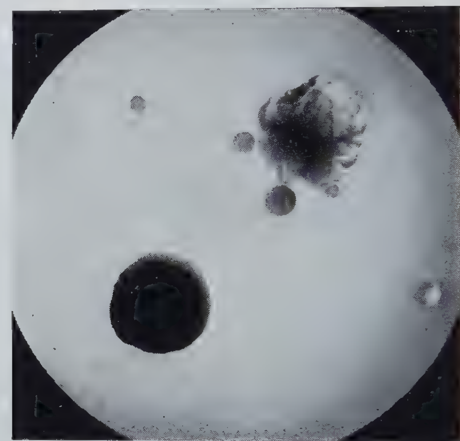

Fig. 5. Heterofermentative *Lactobacillus* sp. Colonies on apple-juice agar. × 20.

Upon plating on YE/glucose/agar, and incubating aerobically, the growth of colonies was very slow, despite their possession of catalase. Two types of colony were produced (a) smooth circular opaque, and (b) rough translucent with root-like protuberances. The smooth opaque colonies frequently gave rise to papillae, or peripheral outgrowths, of the rough translucent colony type. The ratio of smooth opaque to rough translucent colonies was about 2 : 1. Not illustrated (to economise in published space, as with several other strains).

5. *Gluconobacter suboxydans*, strain H–15. From Prof. T. Asai, University of Tokyo. We regard this as a strain of *Acetomonas*, and although it is an acetic acid bacterium, it was included in this study on account of its abundant production of calcium 5–ketogluconate on slopes of YE/glucose/$CaCO_3$/agar, the growth becoming rapidly encrusted with a thick crystalline mass of this compound after only a few days' incubation at 27° C.

Upon plating on YE/glucose/agar 2 distinct colony forms were

obtained (Fig. 6), which we will refer to as 'dark' and 'pale'. Upon picking these separately and growing on slopes of YE/glucose/$CaCO_3$/agar, the dark colonies yielded a growth rapidly becoming encrusted with calcium 5-ketogluconate; the pale colonies did not become thus encrusted, but a slow (7 day's) production of a few large crystals of the same salt took place in the depths of the agar medium.

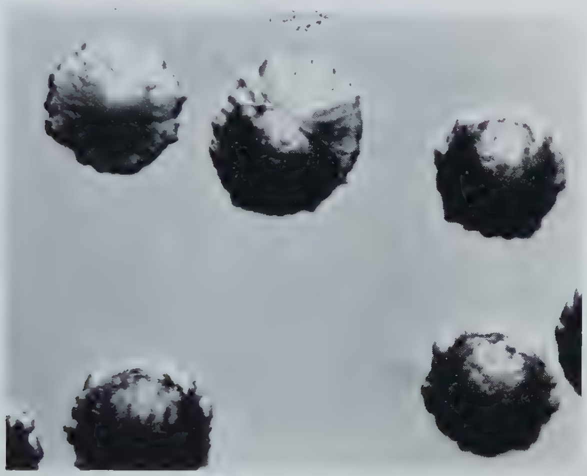

Fig. 6. *Gluconobacter suboxydans*. Colonies on YE/glucose/agar. × 40.

6. *Serratia marcescens*. Although this species is well known for its free production of pigmentation mutants, it was included in this study because it was available as a laboratory stock culture, and the strain was notable for its exceptionally deep blood-red colour.

When plated on YE/glycerol/agar scarcely any two colonies were of the same colour, contour, opacity, and general shape. Some of the more important differences included pure white colonies, pale pink, orange, orange–red, dark red, magenta, and even reddish–sepia. Upon replating white colonies these gave rise to a proportion of pigmented colonies, again of various tints and shapes, including 'variegated' colonies and colonies with a deep red centre and a broad white fringe.

It was found, by continued selection and replating of the deepest red colonies, that eventually an apparently stable red culture was obtained. But, even on replating this, a great variety of colony

shapes and tints were found, and it was noticeable that the deeper the red colour the smoother the type of colony.

As regards the texture of colonies, the deep-red and magenta ones were buttery, whilst many of the white colonies were intensely viscid. Owing to the enormous number of colony forms and the necessity for colour photography, this strain is not illustrated.

7. *Clostridium sporogenes.* (From the collection of Miss J. Davey, Long Ashton). Upon plating this on Difco tryptone/YE/soluble starch/agar, and incubating anaerobically in a McIntosh and Fildes' jar overnight (for 18 hours) at 37° C., a mixture of large colonies of two types were obtained (Fig. 7). The biochemical and other possible differences between the two widely different colony forms were not investigated.

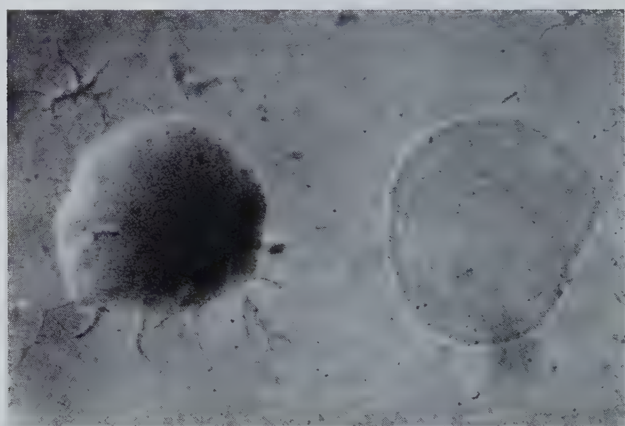

Fig. 7. *Clostridium sporogenes.* Colonies on tryptone/YE/starch/agar. × 20.

8. *Zymomonas anaerobia* var. *pomaceae.* This unusual bacterium (which produces an almost quantitative yeast-like alcoholic fermentation of glucose) although familiar to us, has been included on account of its rarity in collections.

Upon plating on apple-juice agar anaerobically the culture produced two types of colony: (a) large smooth circular opaque, and (b) minute smooth circular opaque. As the latter always remained minute, even on prolonged incubation, this suggests their need for some nutritional factor unnecessary for the larger colonies. Not illustrated.

9. *Flavobacterium proteus.* (N.C.I.B. 877). This is an unusual bacterium, the only gram-negative one capable of multiplying in competition with actively fermenting yeast in a brewery fermentation.

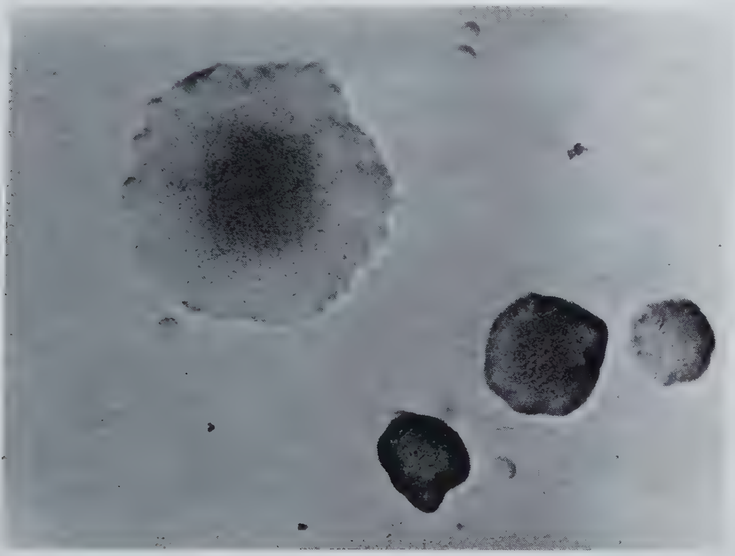

Fig. 8. *Flavobacterium proteus.* Colonies on 'nutrient' agar. × 40.

Nutrient agar plates yielded an immense variety of colony forms, some of which are shown on Fig. 8. The presence of minute circular and rhizoid colonies suggests some nutritional requirement not supplied by the medium, or, alternatively, perhaps, the existence of a life-cycle — a supposition supported by the notorious pleomorphism shown by the cells of this species, notably the existence of large swollen forms.

10. *Achromobacter* species. This was kindly supplied by Dr. James M. Shewan, from the National Collection of Marine Bacteria, in response to our request for a 'typical' *Achromobacter* strain. Upon plating on YE/glycerol/agar, 5 different colony forms were produced after 48 hours at 27° C., namely (1) large smooth opaque circular; (2) irregularly circular, opaque but with coarse granular structure; (3) as (2) but translucent; (4) small granular irregularly circular, with a pale pearly appearance, (5) irregularly circular, almost transparent, with a wavey fringed margin. Not illustrated.

11. *Pseudomonas fluorescens.* Strain 43/2. From the collection of Dr. MURIEL E. RHODES, University of Reading (RHODES, 1959).

When supplying this culture, Dr. RHODES wrote that although the strain was a 'pure culture', in so far as it had been repeatedly purified by replating and picking, it still yielded numerous different colony forms. This we readily confirmed. Fig. 9 shows two of these – dark semi-smooth, and pale rough.

Fig. 9. *Pseudomonas fluorescens.* Colonies on YE/glycerol/agar. × 40.

As this strain was reported to grow solely on ammonium nitrogen we also plated it on Hoyer-Frateur medium (FRATEUR 1950) but using glycerol instead of ethanol. On this medium the dark colonies were only about $\frac{1}{4}$ the diameter of the pale rough ones – suggesting that they had more exacting nutritional requirements, which had been supplied by the previous YE medium. The strain was therefore grown on liquid Hoyer-Frateur medium in serial transfer 3 times. On then replating the final liquid culture on YE/glycerol/agar all colonies produced (60/60) were of the pale rough type shown on Fig. 9, the dark strain having been virtually diluted to extinction by the superior growth rate of the pale rough-type cells in the absence of growth factors. Thus this strain, although originally apparently a prototroph, capable of growing on mineral-nitrogen media, consisted of a mixture of prototrophs and auxo-

trophs, and could be changed the one to the other if the growth-factors in yeast extract were withheld.

Discussion.

In considering the significance of the above results, our premise is that as every one of the 11 different randomly selected strains from 10 different randomly selected genera (*i.e.* 100%) have shown a frequency of occurrence of colony mutants as high as that previously encountered in *Acetobacter*, then it is reasonable to suppose that a similar mutant frequency may well exist in most, if not all, strains of other genera. We submit that if this premise be unacceptable then it follows that we have again (as with *Acetobacter*) unwittingly selected, at random, strains and genera virtually unique in their mutability. This, to us, seems statistically improbable.

If our premise is acceptable, then it seems to follow that most, if not all, pure cultures of bacteria are probably mixtures of cells with different properties. And, furthermore, the different constituent strains of such mixtures will almost certainly not find a change of medium equally favourable to the growth and multiplication of each one of them separately, so that a change of medium (as is necessary for the so-called 'characterization' of a species) will almost certainly result in a change of the proportion in which the constituent cells will exist after growth in the new medium. Indeed such a change of medium (particularly after serial transfer) may well result in the disappearance of all except one of the various cell types in the original culture – and that one not necessarily the one which originally predominated.

The case of the *Pseudomonas fluorescens* strain recorded above indicates, for example, that although the culture as a whole behaved as a 'prototroph', growing on mineral nitrogen, yet it was actually only a proportion of the cells which did so, the 'auxotrophs' or 'semi-auxotrophs' rapidly becoming extinct if the whole culture were grown on mineral-nitrogen media.

Similarly, in *Acetobacter*, even a change in the physical nature of the environment sometimes rapidly resulted in an actual change of 'species' (Shimwell 1956). It has since been found that when a culture, reacting as a whole as *Acetobacter xylinum* when in static culture of organic nitrogen plus ethanol, was transferred to a portion of the same medium but using submerged

aeration with consequent agitation, it was not long before the *A. xylinum* cells became replaced by those of *A. mesoxydans*, which in turn were eventually replaced by those of *A. rancens*.

If it is argued that a heritable change of colony form is nothing more than that, then there is against this argument the weight of opinion and evidence in the literature of bacterial mutation (quite apart from our own findings in most of many *Acetobacter* strains studied). Thus BRAUN (1953) states unequivocally that, 'Undoubtedly most of the smooth, rough, mucoid, etc. changes are in the nature of a biochemical mutation', and supports this statement with numerous examples from the literature.

We have not attempted, in the course of this investigation, to determine in what way(s) all the various mutant colony forms encountered in each strain were correlated with other characters, for experience has shown us that a comprehensive study of even a single new mutant colony is a formidable task. For the correlated change or changes accompanying such colony mutation may require the investigation of, *e.g.*, sugar fermentations, nutritional requirements (including those for numerous vitamins, amino acids, nitrogen-sources, pH etc.) whilst the mutant may also be different from the parent serologically, or in antibiotic resistance, or pigmentation, and so on.

Furthermore, as indicated previously, the study of an organism under such different physical or chemical conditons may result in a partial or complete change of organism, so that in the course of such an investigation one may finish by not studying the original mutant at all. Indeed, it is difficult to escape the conclusion that the terms "species" or "variety" may have little meaning except in relation to a particular environment; change the environment and one may well change the "species".

In view of the now widely acknowledged existence of mutation in so many different species and genera of bacteria in the literature, it is a matter for wonderment why it has made so little impact on taxonomy. We suggest (and this is the main theme of this paper) that this may be due to the fact that the mutation rate per cell per generation (as distinct from mutant frequency) is usually numerically very low, except in one or two characters such as, *e.g.*, S to R type, where the mutation rate is sometimes relatively high (*e.g.* about 5×10^{-2}). We suggest that the commoner mutation rates of between 1×10^{-6} and 1×10^{-10}

(recorded rates for many characters) may have been considered too small to have any material effect on the characters or stability of a 'species', and consequently on its classification.

If so, then it has been overlooked that it is not, *per se*, the mutation rate, but the frequency of occurrence of mutants in a so-called 'pure' culture (*i.e.* how much of a mixture it is) which determines whether it may be usefully classified specifically. If our premise, as previously expressed, is accepted, then it seems that existing classifications, based on the study of cultures under different chemical and physical conditions, may be nothing more than the classification of mixed or composite cultures, which vary in cell composition according to the tests applied.

We are not necessarily suggesting that there is a 'continuous spectrum' of combinations of properties running through the world of bacteria; for although some genera are artificially based on largely utilitarian criteria (such as lactose-fermentation or plant pathogenicity) the members of many groups of bacteria (whether at present segregated in 'genera' or not) do seem to show a close natural kinship, indicating that there is probably a limit, in each direction, to the possible variation or mutation of the properties of the particular group, the boundaries of which define the 'genus'. However, even here some unexpected boundaries have been crossed. For instance, as mentioned under 'Introduction', the genus *Acetobacter*, with its 'characteristic' property of oxidizing ethanol to acetic acid, has been found to contain strains which can lose this essential generic character, thereupon becoming unrecognisable as acetobacters, although retaining other properties of their parent *Acetobacter* strains (SHIMWELL and CARR, 1960).

Whilst granting that the high mutant frequency in the 11 randomly selected strains recorded above, together with the same degree of mutant frequency in some 100 or more *Acetobacter* strains previously studied, may have been fortuitous, yet we think this statistically so unlikely that if those specializing in other genera should similarly examine their cultures, a similarly high mutant frequency might well be encountered, with consequent repercussions on taxonomy.

Finally, we emphasize that whilst a 'colony mutation' is usually, if not always, 'in the nature of a biochemical mutation' (BRAUN, 1953), the converse does not necessarily apply, many changes in biochemical properties not resulting in a change of colony form. For example, some 'quasi-acetobacters' (SHIMWELL and CARR, 1960)

although they had lost the important property of acetifying ethanol, were not distinguishable from acetobacters by colony appearance. Similarly the literature contains many instances of lack of correlation between the changed biochemical properties of a mutant and its colony form. Thus the mutants detected visually in the course of this investigation probably represent only a proportion of those present in the cultures. If so, the same might well apply in other genera.

Summary.

Every one of eleven different strains randomly selected from 10 different randomly selected genera have shown the same high frequency of occurrence of colony mutants as did almost all strains of *Acetobacter* (previously considered outstanding in this respect). Correlation of other properties with such mutant colony forms was not specifically studied, but in 4 strains correlation was noticed, suggesting its presence in the others, as was so often found in *Acetobacter*.

It is suggested from this, that a similar study of strains of other genera might reveal a similarly high frequency of occurrence of mutants, most so-called pure cultures being thus probably mixtures of different cells with different properties. Also the proportion of each cell-type in the culture may vary from predominance to extinction according to the biochemical and other tests applied for the purpose of the 'characterization' of the species for taxonomic purposes.

If the classification of such varying mixtures is considered of doubtful use, then it seems to follow that 'species' of bacteria are virtually unclassifiable, and that even the conception of a genus should be on a broader basis than is often the case at present.

Acknowledgement.

We wish to thank Miss J. Davey for kindly preparing the plates of *Clostridium sporogenes* for photography.

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POLIOMYELITIS ANTIBODIES IN SERA FROM THE NETHERLANDS, CURAÇAO, SURINAME, ST. EUSTATIUS AND NETHERLANDS NEW-GUINEA [1] [2]

by

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(Received June 3, 1960).

INTRODUCTION.

The sera for this survey were collected before the large-scale introduction of poliomyelitis vaccines in the Netherlands and Curaçao.

This study gave us an opportunity to compare the results from the Netherlands with those from other parts of the Kingdom. Moreover the data from the Netherlands might confirm the fact, that there is a close correlation between the morbidity of poliomyelitis and the immunity status of a population.

Together with the large number of other communications on this subject from different parts of the world (PAUL and RIORDAN 1950; PAUL, RIORDAN and MELNICK 1951; PAUL, MELNICK, BARNETT and GOLDBLUM 1952; McD. HAMMON and SATHER 1953; PAUL and HORSTMANN 1955; BARSKI and LÉPINE 1956; DANE, DICK, CONOLLY, BRIGGS and McLEOD 1956; FOX, GELFAND, LEBLANC and CONWELL 1956; GELFAND and MILLER 1956; LAPINLEIMU 1956; BERNKOPF, MEDALIE and YEKUTIEL 1957; DEBRÉ, CELERS,

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[2]) With technical assistance of Mr. P. LAMAN, Mrs. O. J. A. MEYBURG-CALMEYER, Miss C. WESTENBROEK and Miss A. DE HAAN.

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Drouhet and Soulé 1957; Quersin-Thiry, de Somer, Nihoul and Prinzie 1957; Wohlraab, Höpkens and Anz 1957; Kono *et al.* 1958; Tatsumi 1958) these results may add to the information about the status of immunity against poliomyelitis.

Materials and methods.

The 4155 sera were collected from:

The Netherlands	2073 sera
Curaçao	1669 sera
Suriname	46 sera
St. Eustatius	20 sera
Netherlands New-Guinea	347 sera

In the Netherlands the sera were collected from children's hospitals or from the outpatients wards[1]). Children with infectious diseases or neurological disorders were excluded. Adult sera from the Netherlands were acquired from the Central Laboratory of the Bloodtransfusion Service of the Netherlands Red Cross, Amsterdam (Prof. Dr. J. J. van Loghem Jr.) and from outpatient wards in hospitals[1]) (persons with infectious diseases excluded).

The sera from Curaçao and St. Eustatius were received from Dr. C. Winkel and Dr. J. G. A. Borghans; the Central Laboratory of the Bloodtransfusion Service of the Netherlands Red Cross, Amsterdam sent us sera from Suriname and Dr. J. C. Vogel (Hollandia) and Dr. R. A. de Haas (Ifar) provided us with sera from Netherlands New-Guinea.

We did not have at our disposal information about family size, social status etc.; therefore we were not able to compare our results with those of Noordam *et al.* (1959). Sera were stored at —20° C.

Antibodies were determined in a slightly modified color-test (Salk, Youngner and Ward (1954)).

The sera were diluted twofold from 1/4 to 1/512 in tubes of 55 × 10 mm. To each tube containing 0.25 ml serumdilution were added ± 100 $TCID_{50}$ of poliovirus, type I, II and III respectively in a 0.25 ml volume. Serum-virus mixtures were held at room temperature for two hours. Thereafter cells (trypsinized monkey kidney cells 250,000/ml) were added to the mixtures, 0.25 ml pro tube, and tubes were sealed off with rubber stoppers or mineral oil.

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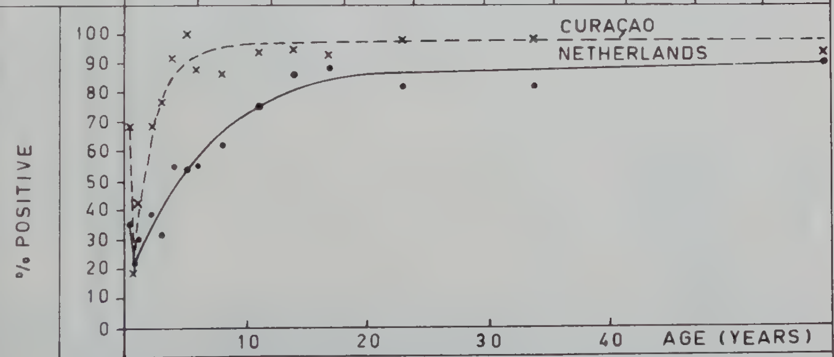
[1]) We thank all those, who cooperated to make these sera available to us.

TABLE 1 SERA CONTAINING ANTIBODIES AGAINST POLIOMYELITIS VIRUS TYPE I

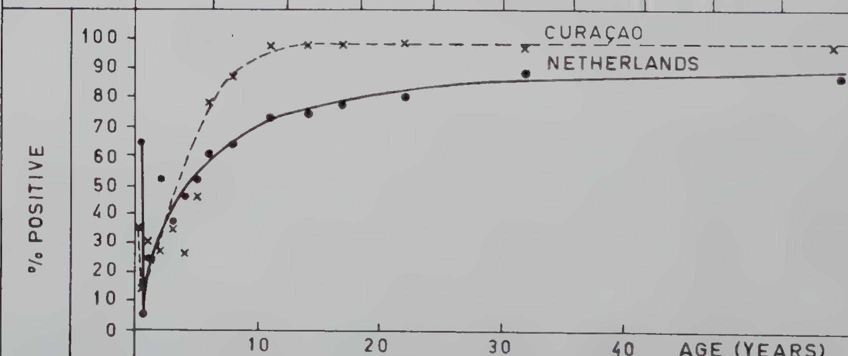
AGE (YEARS)	NETHERLANDS		CURAÇAO		SURINAME		ST EUSTATIUS		NW GUINEA	
	x)	%	x)	%	x)	%	x)	%	x)	%
0 – ½	4/11	36	24/35	69	–	–	–	–	0/1	–
½ – 1	3/14	21	7/38	18	–	–	–	–	4/4	–
1	19/63	30	33/77	43	–	–	3/7	–	32/68	47
2	33/85	39	18/26	69	–	–	4/6	–	27/62	44
3	29/94	31	13/17	76	–	–	0/2	–	3/4	–
4	63/114	55	10/11	91	–	–	1/1	–	77/94	82
5	70/129	54	9/9	100	–	–	4/4	–	1/2	–
6	57/104	55	8/9	89	–	–	–	–	3/3	–
7, 8, 9	192/314	61	36/42	86	4/6	–	–	–	4/5	–
10, 11, 12	191/253	75	111/118	94	14/15	93	–	–	5/5	–
13, 14, 15	68/80	85	93/98	95	2/2	–	–	–	3/4	–
16, 17, 18	43/49	88	118/128	92	1/2	–	–	–	5/5	–
19 – 25	157/195	81	206/210	98	8/8	–	–	–	44/47	94
26 – 40	237/291	81	314/321	98	6/7	–	–	–	31/35	89
> 40	250/277	90	490/530	92	4/6	–	–	–	8/8	–



x) NUMERATOR = NUMBER OF SERA CONTAINING ANTIBODIES
DENOMINATOR = NUMBER OF SERA TESTED

TABLE 2 SERA CONTAINING ANTIBODIES AGAINST POLIOMYELITIS VIRUS TYPE II

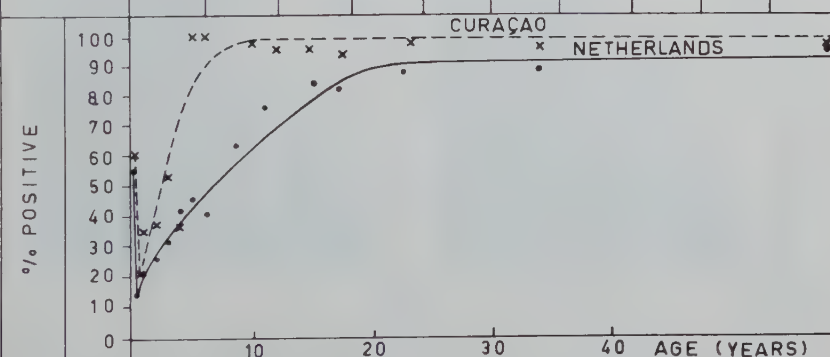
AGE (YEARS)	NETHERLANDS		CURAÇAO		SURINAME		ST. EUSTATIUS		NW. GUINEA	
	x)	%	x)	%	x)	%	x)	%	x)	%
0–½	7/11	64	12/35	34	–	–	–	–	0/1	–
½–1	1/14	7	5/38	13	–	–	–	–	2/4	–
1	15/63	24	23/77	30	–	–	0/7	–	20/68	29
2	43/85	51	7/26	27	–	–	0/6	–	21/62	34
3	36/94	38	6/17	35	–	–	0/2	–	0/4	–
4	54/114	47	3/11	27	–	–	0/1	–	58/94	62
5	67/129	52	4/9	44	–	–	1/4	–	1/2	–
6	62/104	60	7/9	78	–	–	–	–	3/3	–
7, 8, 9	203/314	64	36/42	86	6/6	–	–	–	4/5	–
10, 11, 12	183/253	72	116/118	98	15/15	100	–	–	5/5	–
13, 14, 15	59/80	74	96/98	98	2/2	–	–	–	2/4	–
16, 17, 18	38/49	77	126/128	98	2/2	–	–	–	5/5	–
19–25	154/195	80	208/210	99	8/8	–	–	–	43/47	91
26–40	259/291	89	310/321	96	6/7	–	–	–	34/35	97
> 40	244/277	88	515/530	97	3/6	–	–	–	8/8	–



x) NUMERATOR = NUMBER OF SERA CONTAINING ANTIBODIES
DENOMINATOR = NUMBER OF SERA TESTED

TABLE 3 SERA CONTAINING ANTIBODIES AGAINST POLIOMYELITIS VIRUS TYPE III

AGE (YEARS)	NETHERLANDS		CURAÇAO		SURINAME		ST. EUSTATIUS		NW. GUINEA	
	x)	%	x)	%	x)	%	x)	%	x)	%
0–1/2	6/11	55	21/35	60	–	–	–	–	0/1	–
1/2–1	2/14	14	8/38	21	–	–	–	–	2/4	–
1	13/63	21	27/77	35	–	–	0,7	–	60/68	88
2	22/85	26	10/26	38	–	–	1/6	–	24/62	39
3	29/94	31	9/17	53	–	–	1/2	–	3/4	–
4	47/114	41	4/11	36	–	–	1/1	–	61/94	65
5	58/129	45	9/9	100	–	–	4/4	–	2/2	–
6	42/104	40	9/9	100	–	–	–	–	2/3	–
7, 8, 9	197/314	63	41/42	98	5/6	–	–	–	5/5	–
10, 11, 12	192/253	76	112/118	95	12/15	80	–	–	5/5	–
13, 14, 15	67/80	84	93/98	95	2/2	–	–	–	3/4	–
16, 17, 18	40/49	82	119/128	93	2/2	–	–	–	5/5	–
19–25	172/195	88	206/210	98	7/8	–	–	–	45/47	96
26–40	259/291	89	310/321	96	7/7	–	–	–	35/35	100
>40	263/277	95	517/530	97	4/6	–	–	–	8/8	–



x) NUMERATOR = NUMBER OF SERA CONTAINING ANTIBODIES
DENOMINATOR = NUMBER OF SERA TESTED

All dilutions and the virus- and cellsuspensions were prepared in a medium containing 0.5% lactalbumin hydrolysate and 5% horse-serum in a buffersolution (Hanks). Antibiotics and mycostatin were added to the medium.

After one week incubation at 36° – 37° C. the test was read. Titers were expressed as highest serum dilutions showing a yellow colour in the tubes. If there was no change of colour to yellow in the tubes containing the 1/4 serum dilutions, these sera were considered negative.

Results.

For each age group the number of sera containing antibodies against each of the three polio types was determined. The results are presented in tables 1, 2 and 3.

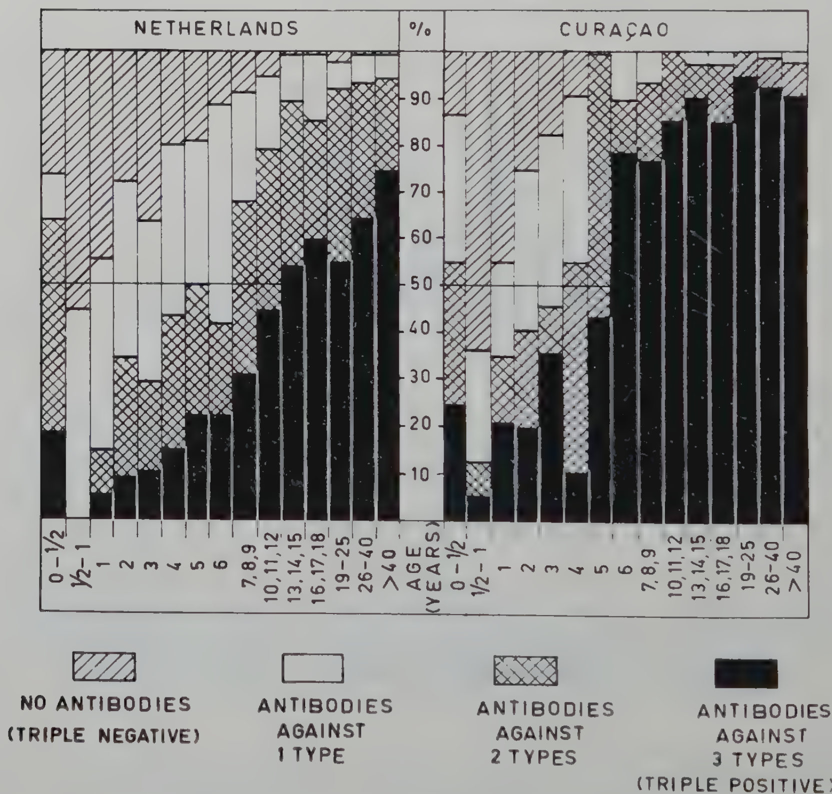


Fig. 1. Percentage distribution according to age of sera without antibodies and with antibodies against one or more types of the poliomyelitis virus.

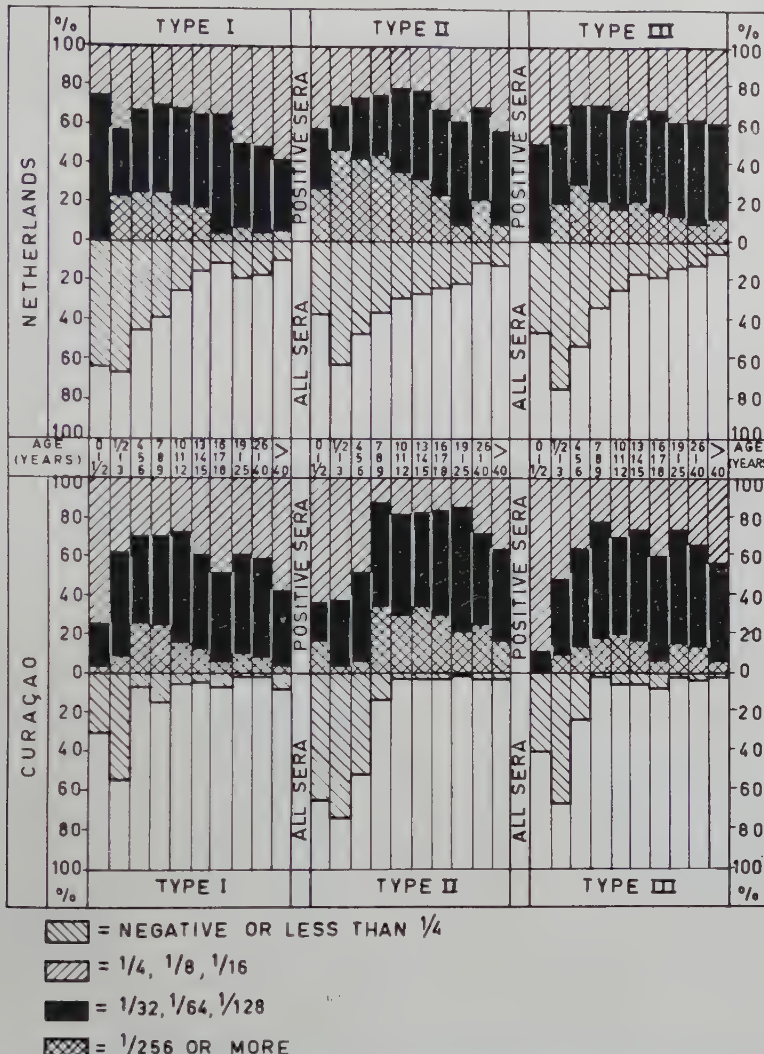


Fig. 2. Frequency distribution of antibody-levels according to age in the sera from the Netherlands and Curaçao.

These results show, that infection with poliomyelitis virus in Curaçao takes place at an earlier age than in the Netherlands. The curves for type I are the most clear in this respect. From Suriname, St. Eustatius and New-Guinea we did not have enough material at our disposal for a clear evaluation. It looks, as if these sera show the same pattern as those from Curaçao, which points to

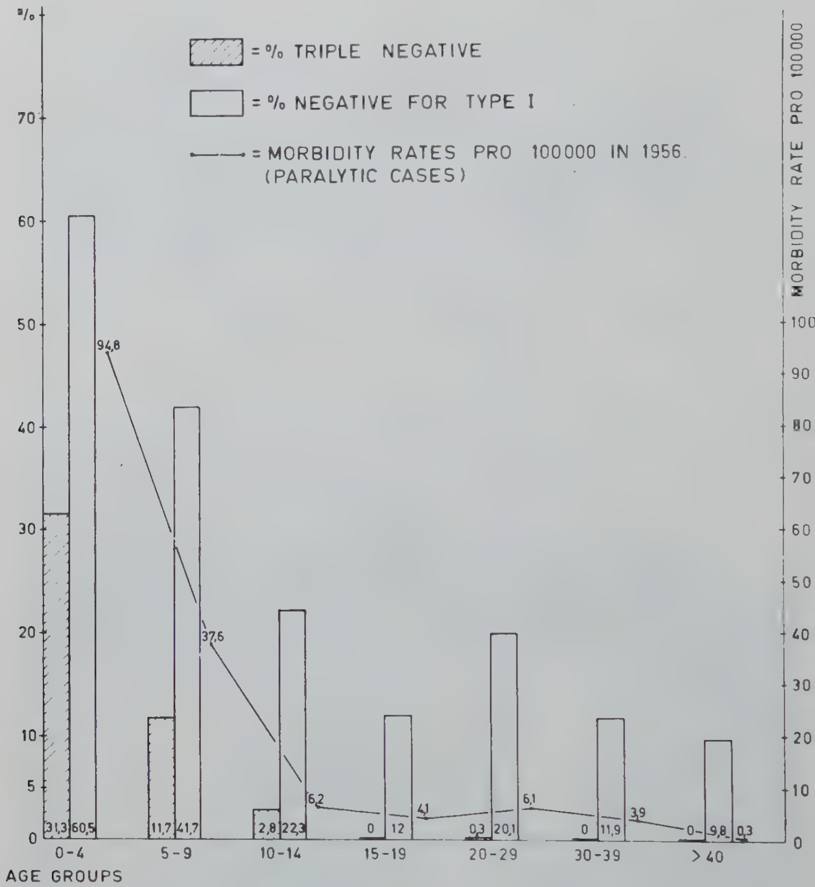


Fig. 3. Percentages of sera without antibodies against three types, without antibodies against type I and of morbidity rates in 1956 in different age groups in the Netherlands.

infection in early childhood. Only the results from St. Eustatius for type II antibody might suggest, that on this island contact with type II poliomyelitis virus occurs later.

Considering the three curves from the Netherlands it appears, that the slope for type III levels off sooner than for type I and type II. Possibly infection with type III takes place somewhat later than with the other two types.

Figure 1 shows the frequency distribution of sera with antibodies against one, two or three types and that of triple negative sera.

The pattern for Curaçao, Suriname, St. Eustatius and New-Gui-

nea points to an earlier and more extensive infection in these countries than in the Netherlands. This can be shown clearly, by determining the age, at which 50% of the population is triple positive. For the Netherlands this is ± 12 – 13 years, for Curaçao Suriname and New-Guinea 5 – 6 years.

In the sera from Curaçao and the Netherlands the amount of antibody was determined. The sera were divided in three groups

1. with a high titer (more than 1/256).
2. with a medium titer (1/32, 1/64 and 1/128).
3. with a low titer (1/4, 1/8 and 1/16).

In figure 2 the relative amounts of high, medium and low titered sera are presented according to age.

It is obvious that among positive sera high titers are most frequent in the younger age groups, where the first infection with the poliovirus takes place.

Discussion.

From the results presented above it is evident that there is a different pattern of infection with poliomyelitis virus in the Netherlands and the other parts of the Kingdom (Curaçao, St. Eustatius, Suriname and New-Guinea). The latter group shows a pattern, similar to that in Cairo (Paul *et al.* 1952) and in other tropical and subtropical areas (McD. Hammon and Sather 1953; Paul and Horstmann 1955; Barski and Lépine 1956; Gelfand and Miller 1956; Kono *et al.* 1958).

The data from the Netherlands point to a similarity with other countries in the temperate zones of the Northern Hemisphere. Moreover it could be confirmed for the Netherlands that there is a good correlation between the serological pattern and morbidity, fig. 3. (Verslagen en mededelingen Volksgezondheid 1958, pag. 1144).

In the older age groups we found in all geographical areas a gradual diminution of the number of persons with high antibody titers. This has also been reported by others (Le Bouvier 1957).

Summary.

Poliomyelitis antibodies were determined in 4155 sera from the Netherlands, Curaçao, Suriname, St. Eustatius and New-Guinea.

The results showed that in the Netherlands the serological pattern for poliomyelitis was different from that in the tropical areas Curaçao, Suriname, St. Eustatius and New-Guinea, which points to infection with poliomyelitis virus at an earlier age in these latter parts of the world.

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RECHERCHES SUR LE MECANISME D'ACTION DE *BACILLUS THURINGIENSIS*

EFFET DE LA TOXEMIE SUR LES FRACTIONS PROTEIQUES DE L'HEMOLYMPHE

par

A. DRILHON et **C. VAGO.**

(Reçu le 28 avril, 1960).

Plusieurs souches bactériennes du groupe *Bacillus cereus* isolées de Lépidoptères se distinguent par une action pathologique particulièrement virulente vis à vis de nombreux insectes. Ces souches (2, 3, 13, 15) rapportées sous les noms *thuringiensis* Berliner, *sotto* Ishiwata, *alesti* Toumanoff et Vago, sont caractérisées par la formation d'inclusions cristallines auxquelles est lié un effet toxique (1, 8). De telles bactéries (*sotto*, *alesti*) provoquant en sériciculture des actions destructives, sont par ailleurs de plus en plus utilisées dans la lutte microbiologique contre les insectes nuisibles. Aussi de nombreux travaux se consacrent-ils depuis quelques années à ces germes, lesquels ont été séparés du groupe *cereus* à cause de leur nature cristallophore et réunis dans le groupe des *thuringiensis* (9).

En dehors des aspects économiques, ces bactéries présentent un intérêt en pathologie comparée à cause de leur mode d'action particulier ne pouvant pas être identifié à des mécanismes d'effets toxiques connus chez les bactéries sporulées. Plusieurs travaux essayent d'analyser cette action, surtout ces dernières années, aussi bien par la séparation des cristaux toxiques que par des tentatives de localisation de l'attaque des tissus dans l'organisme des Lépidoptères (8, 10, 14). D'une façon générale, on reconnaît l'existence d'un effet toxémique qui suit immédiatement, chez certains Lépidoptères, l'ingestion du germe et d'une action liée à la multiplication des formes végétatives de la bactérie. Cependant, même dans ce deuxième

cas, une préparation du terrain par une certaine toxicité est supposée (14).

Malgré ces études, l'effet physiologique de la substance toxique n'est pas connu. Nous avons certains renseignements sur la composition des cristaux au point de vue protéinique (8). En ce qui concerne le mode d'action toxique lui-même, les recherches sont à leurs débuts. Une augmentation de l'alcalinité de l'hémolymphe au cours de la toxémie a été signalée (10). D'un autre côté, en 1952, nous avions pu mettre en évidence la disparition d'un certain nombre d'acides aminés essentiels ainsi que d'importantes quantités de substances fluorescentes: flavine, pterines, acide folique. Les phénomènes sont concomitants de l'état paralytique installé (arrêt des mouvements stade 3 et stade 4 de l'infection) (7). L'électrophorèse sur papier accusait une diminution de 45% environ de la valeur respective des fractions séparées, à ces mêmes stades (5).

En vue d'approfondir l'aspect physiologique de la toxémie nous avons poursuivi l'analyse d'hémolymphe d'insectes atteints de *Bacillus thuringiensis* var. *alesti* par la technique d'électrophorèse en gel d'amidon. En effet, cette méthode apporte un pouvoir sélectif remarquable à la séparation de diverses globulines et on sait qu'ici au phénomène électrocinétique vient s'ajouter une séparation de grosses molécules d'avec les plus petites ayant cependant une même charge électrique.

Des larves de *Bombyx mori*, 48 h. après la sortie de la quatrième mue et après un jeûne de 6 heures reçoivent un repas de feuilles trampées dans une suspension aqueuse de culture (6 jours) de *B. thuringiensis* var. *alesti* dosant environ 1.000.000 de spores par cc. Au bout de 30 minutes les feuilles sont retirées et une prise de sang est effectuée aux divers stades de la progression de la maladie. Nous avons distingué les quatre catégories suivantes:

Type 1: Témoin sans infection, sans signe de paralysie.

Type 2: Apparition des premiers signes de crampes (environ 3 h. après l'infection).

Type 3: Paralysie partielle, incapacité de déplacement (de 3 à 5 h. après l'infection).

Type 4: Paralysie totale, seule les vraies pattes tremblent (de 5 à 8 h. après l'infection).

L'hémolymphe prélevée sur 10 larves de chaque catégorie est mélangée et centrifugée à 4.000 t/m pendant 10 minutes afin d'éliminer les éléments figurés. Les examens sont effectués sur les liquides clairs ainsi obtenus.

Nous avons suivi la teneur en protéine totale de l'hémolymphe au cours des différents stades de l'infection (16). Voici la moyenne des résultats obtenus:

Type 1 : 68,2 gr. ‰.
Type 2 : 52 gr. ‰.
Type 3 : 49 gr. ‰.
Type 4 : 38,5 gr. ‰.

L'électrophorèse sur papier nous avait permis de suivre l'évolution des fractions protéiques – au cours de la croissance de la larve de *B. mori* (5) et au cours de la métamorphose de celle-ci. A la fin du 5ème âge larvaire, on parvenait à séparer au maximum trois fractions globuliniques, de poids moléculaires élevés, comme l'ont montré ODA, KAYASHI et SASAKI (11). Le papier utilisé comme support, ou le tampon véronal, paraissent convenir mal à une séparation fine des fractions globuliniques.

C'est pourquoi nous avons repris cette étude, sur gélose et sur amidon (6,7). Parallèlement à nous, DENUCE (4) publiait les images données par le *Bombyx mori* normal au cours d'électrophorèses menées à bien sur ce support. Au cours du 5ème âge larvaire, âge des larves témoins de nos infections expérimentales, il était aisé de dénombrer de 11 à 12 fractions différentes.

Si dans l'état actuel de nos connaissances, on ne puisse faire une corrélation certaine entre ces fractions individualisées, et celles que l'on commence à bien connaître chez l'homme, il n'en demeure pas moins certain, que quantitativement, l'aspect de la migration se modifie considérablement au cours de l'infection, et qu'un certain nombre de fractions disparaissent même complètement. Ces résultats viennent compléter et amplifier ce que nous savions des acides aminés. En particulier, certaines fractions comme les gamma globulines qui seules migrent du côté cathodique sont parfaitement identifiées. Elles apparaissent au cinquième stade larvaire; deux fractions, une lente et une rapide sont mises en évidence. Au stade terminal de la paralysie il ne reste plus qu'une seule fraction γ 1 (Fig. 1).

Il paraît y avoir une pré-albumine chez la larve normale et une ou plusieurs fractions albumine. Au cours de la toxémie, toutes les fractions intermédiaires entre une faible fraction d'albumine et une fraction à grosse affinité tinctoriale que l'on suit depuis le début de la vie larvaire ont disparu. Les fractions demeurant sont quantitativement moindres par rapport au témoin.

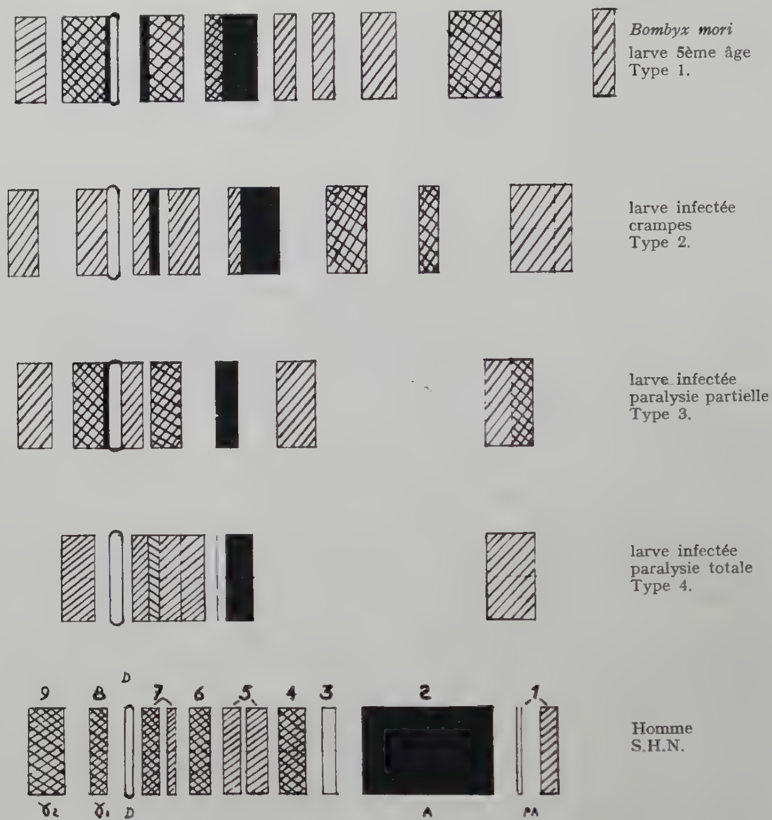

Fig. 1. Fraction d'hémolymphe de *Bombyx mori* sain et en voie de toxémie séparée par électrophorèse sur amidon.

Les différentes fractions séparées dans un sérum humain normal (S.H.N.) correspondent aux appellations suivantes, en suivant le gel de l'anode à la cathode.

1 – Une ou deux pré-albumines (PA).

2 – La bande des albumines (A).

3 – La post albumine.

4 – La bande des $\alpha 2$ (rapides ou fast $\alpha 2$).

5 – La bande des $\alpha\beta$.

6 – Les β globulines.

7 – Les $\alpha 2$ lentes ou slow $\alpha 2$.

D – La tranchée d'insertion.

8–9 – Les γ globulines (du côté cathodique).

L'un des aspects de la toxémie est représenté par la suppression du phénomène de la mélanogénèse. Le noircissement de l'hémolymphe des larves de *B. mori* au cinquième âge est normalement très prononcé et l'hémolymphe en contact avec l'air prend une couleur noire au bout de 30 minutes à 2 heures. Par contre, le sang prélevé au stade de crampes (type 2) n'atteint pas cette couleur et prélevé au début de la paralysie (type 3) il brunit à peine pour rester parfaitement clair (type 4) lorsqu'il provient d'un insecte totalement paralysé (Fig. 2).

Fig. 2. Progression de l'inhibition de la mélanisation de l'hémolymphe chez *Bombyx mori* au cours de la toxémie à *Bacillus thuringiensis* var. *alesti*. De gauche à droite: hémolymphe normale, paralysie type 2, type 3 et type 4.

Ce phénomène est vraisemblablement expliqué par la disparition des acides aminés nécessaires à la mélanogénèse: phénylalanine, tyrosine ou de substances enzymatiques comme les polyphénol-oxydases.

Les modifications relatées dénotent une action profonde de la toxémie sur la composition chimique de l'hémolymphe. La diminution si importante et si rapide des acides aminés et des fractions

protéiques paraît susceptible d'amener des troubles dans les échanges sanguins ou des modifications tissulaires pouvant s'extérioriser par le syndrome de la paralysie progressive. On pourrait rechercher certaines relations avec les résultats récents de H. SHIGEMATSU (12), qui paraît avoir démontré la synthèse des protéines de l'hémolymphe du *Bombyx* au sein du tissu adipeux. Une atteinte de celui-ci par la toxine, serait alors en rapport avec les modifications observées. Toutefois des recherches s'imposent au sujet de la compatibilité de la rapidité d'action avec un dérangement au niveau de la synthèse.

L'ensemble des changements reflète plutôt une action lytique directe ou indirecte de la toxine dissoute, peut-être en rapport étroit avec l'augmentation de l'alcalinité de l'hémolymphe observée au cours de la toxémie.

C'est sur ce point que portent nos recherches en cours.

Résumé.

Par l'analyse des protéines totales et l'électrophorèse sur gélose et sur amidon de l'hémolymphe de *B. mori* (Lépidoptère), des modifications profondes sont apparues au cours de la progression de la toxémie due à *Bacillus thuringiensis* var. *alesti*. Une partie des acides aminés et des fractions protéiques diminue au fur et à mesure que la paralysie s'installe et certaines fractions tendent à disparaître. Ce processus est précisé pour chacun des éléments séparés.

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(From the National Institute of Public Health, Utrecht, Netherlands).

DETERMINATION OF DIPHTHERIA AND TETANUS ANTITOXIN WITH THE AID OF HAEMAGGLUTINATION

by

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During the past few years many investigators (*e.g.* Boyden, 1951; Fisher, 1952; Stavitsky, 1954; Landy, 1955; Scheibel, 1956, and Fulthorpe, 1957, 1958, 1959) have concerned themselves with the development of a method of determining diphtheria and tetanus antitoxin titres in human, guinea-pig and horse sera by means of haemagglutination titration. This haemagglutination technique might offer considerable advantages in that experiments in guinea-pigs and mice would become superfluous, while the titres would be known within a day. Titres determined by animal experiments are not available until after two or four days.

Although the above mentioned authors obtained reasonable results, the continued publication of reports on the subject indicates that the search for the most reliable method of titration continues. Since diphtheria and tetanus antitoxin titrations are frequently carried out in the Laboratory for Serum and Vaccine Purification, there were reasons for testing the value of the haemagglutination technique so that, if it appeared justifiable, this method of titre determination might be adopted.

Material and methods.

Members of the Institute Staff were requested to provide 100 ml blood each so that all titrations might be made on the sera from these persons, after which the results obtained might be compared; 28 persons volunteered.

Method of titration.

The sera from the volunteers were titrated by the following methods:

I. In-vivo titrations

Diphtheria: determination of antitoxin titre by the intracutaneous method, on the following bases:
$L_r/100$, $L_r/1000$, $L_r/10.000$

Tetanus: determination of antitoxin titre with mice, on the following bases:
$L+/100$, $L+/1000$, $L+/10.000$

II. In-vitro titrations

Diphtheria: determination of antitoxin titre:
a. by the direct haemagglutination method and
b. by the haemagglutination inhibition test on the following bases:
$L_A/10$, $L_A/100$, $L_A/1000$

Tetanus: determination of antitoxin titre:
a. by the direct haemagglutination method and
b. by the haemagglutination inhibition test on the following bases:
$L_A/10$, $L_A/100$, $L_A/1000$.

The diphtheria titrations were carried out by the in-vivo method on the bases $L_r/100$, $L_r/1000$, $L_r/10.000$, the tetanus titrations on the bases $L_+/100$, $L_+/1000$, $L_+/10.000$, and the haemagglutination inhibition test on the bases $L_A/10$, $L_A/100$, $L_A/100C$ in the various titration methods, therefore, the mixtures of serum and toxin (or toxoid) were standardized at 0.1, 0.01 and 0.001 AU antitoxin respectively.

Haemagglutination titrations.

a. Direct method.

Principle: Dilutions in a series of tubes are made from the serum of which the titre is to be determined by haemagglutination. If sufficient antitoxin is contained in the mixture after addition of sheep erythrocytes sensitized with toxoid[1]), this antitoxin causes complete

[1]) for preparation of sensitized erythrocytes see page 416.

agglutination of the sensitized erythrocytes. If the quantity of antitoxin in a given mixture is insufficient to cause complete agglutination of the blood cells, these cells form a smooth ring on the bottom of the tube after a few hours.

Practical experience has shown that the most reliable results are obtained by selecting as the end-point of titration that mixture in which the red cells only just escape clearly visible agglutination.

b. Haemagglutination inhibition test.

Principle: Into a series of tubes a quantity of toxoid is pipetted which, when mixed with 0.1, 0.01 or 0.001 AU antitoxin, according to the level at which serum titration is intended, is barely capable of preventing agglutination of the sensitized blood cells added. This quantity of 'test toxoid' is determined in advance by introducing 0.1, 0.01 or 0.001 AU of a standard serum, into a series of tubes, and mixing these specimens with increasing quantities of toxoid. Having been made up to the same volume with 0.5% normal rabbit serum, each tube is then treated as follows: 0.05 ml of a suspension of sheep erythrocytes sensitized with toxoid is pipetted into it, after which it is shaken for 30 seconds; the resulting agglutination of blood cells is evaluated after a given interval. That mixture in which the blood cells barely escape agglutination is taken as end-point or point of equivalence.

Dilutions are made with a serum to be titrated, and these are mixed with the dose of 'test toxoid' determined in advance and present in the tubes. After addition of 0.5% normal rabbit serum in saline solution up to 1 ml, 0.05 ml of a suspension of sheep erythrocytes sensitized with toxoid is added to each tube; the contents of the tubes are then shaken. If the quantity of serum added contains less antitoxin than is required for neutralization of the test toxoid, then the erythrocytes do not agglutinate but settle to form a smooth ring on the bottom of the tube.

In the presence of an excess of antitoxin the sensitized cells agglutinate and form an irregular deposit of clumped blood cells on the bottom of the tube.

The end-point chosen was that quantity of serum which, when mixed with the quantity of test toxoid added, just failed to cause agglutination of the sensitized blood cells. This quantity of serum, in this dilution, corresponds to 0.1, 0.01 or 0.001 AU antitoxin, according to the level at which titration is carried out. Multiplication

by the dilution factor gives the number of antitoxin units per ml of the titrated serum.

Technique of reading.

Titration readings are made by holding a rack of tubes above a mirror; in this way the reaction of the blood cells can be sharply evaluated in the reflection. Reading is greatly simplified by using a frame holding a swinging mirror. The racks of tubes are placed on this frame, so that several titrations can be simultaneously evaluated in the mirror.

Reagents.

Sterile buffered saline solution;

for 200 ml buffered saline solution pH 6.4:
100 ml saline solution
32 ml $Na_2HPO_4 . 2H_2O$ solution 0.15 mol.
68 ml KH_2PO_4 solution 0.15 mol.

for 200 ml buffered saline solution pH 7.4:
100 ml saline solution
76 ml $Na_2HPO_4 . 2H_2O$ solution 0.15 mol.
24 ml KH_2PO_4 solution 0.15 mol.

A solution of tannic acid in saline solution 1 : 40.000;
to be freshly prepared before use (tannic acid analytic quality 'MERCK').
A solution of 0.5% normal rabbit serum in saline solution;
the rabbit serum to be inactivated in advance for 30 minutes at 56° C.
Purified diphtheria toxoid;
Purified diphtheria serum, standardized against the International Standard, dilution ratio = 1 (GLENNY and LLEWELLYN–JONES, 1931);
Purified tetanus toxoid;
Purified tetanus serum, standardized against the International Standard, dilution ratio = 1.
Preparation of the sensitized sheep erythrocytes.

This was done largely according to the method of SCHEIBEL (1956): 50 ml fresh sheep blood, shaken with glass beads for 10 minutes, is washed four times with 200 ml sterile saline solution and then centrifuged for 7 minutes at 2000 r.p.m. The sediment is resuspended in 200 ml buffered saline solution pH 7.4. The concentration of this erythrocyte suspension is determined with the aid of a haemolysis test. For this purpose, 1 ml of the suspension obtained is mixed with 6 ml distilled water. The colour intensity of the haemoglobin liberated is measured with the Beckman spectrophotometer, 1 ml cuvette, wavelength 500 mμ. There should be 20% transmission measured against distilled water.

200 ml of this erythrocyte suspension are mixed with 200 ml of a solution of tannic acid (1 : 40.000) in saline solution. The mixture is kept at room temperature for 10 minutes and then centrifuged for 7 minutes at 2000 r.p.m.; the sediment is once washed with 200 ml sterile saline solution and resuspended in unbuffered saline solution to 200 ml.

200 ml of the tannic acid-treated cells are mixed with 1000 ml purified tetanus or diphtheria toxoid diluted with buffered saline solution pH 6.4 to 6 Lf/ml. This mixture is kept at room temperature for 10 minutes; it is then centrifuged for 7 minutes at 2000 r.p.m. and the sediment is washed three times with 300 ml 0.5% normal rabbit serum in saline solution. The washed cells are resuspended in 200 ml 0.5% rabbit serum in saline solution, and this quantity is distributed over sterile 10 ml bottles and kept at 4° C. The blood cells thus prepared can be used for 3–4 weeks.

To ensure reliable results in preparing the sensitized cells and in the titrations to be made, it is necessary always to use the same toxoids and standard sera, a sufficient stock of which should be stored in the refrigerator.

Solutions for immediate use having low toxoid or serum concentrations, *e.g.* 10 Lf or 10 AU per ml, can be prepared by diluting with saline solution.

Procedure of the direct haemagglutination method.

With a slight modification, the dilution method of HORSFALL and TAMM (1953) was employed in the procedure of the direct method.

The serum to be titrated is inactivated for 30 minutes at 56° C. and then diluted 10 × with 0.5% normal rabbit serum in saline solution, to prevent aspecific reactions.

Into three bottles (A, B and C), the following quantities of diluted serum and 0.5% rabbit serum in saline solution are then pipetted:

bottle A: 1.75 ml diluted serum and 0.45 ml 0.5% rabbit serum
bottle B: 1.35 ml diluted serum and 0.79 ml 0.5% rabbit serum
bottle C: 0.95 ml diluted serum and 0.90 ml 0.5% rabbit serum.

The sera in the bottles have now been diluted 12.6, 15.85 and 20 times respectively; in logarithms: 1.1, 1.2 and 1.3 respectively.

Into three series of 12 tubes – series A, B and C – 0.5 ml 0.5% rabbit serum each is pipetted, with the exception of the first tube of each series. From bottle A, 0.5 ml each of diluted serum is pipetted into the 1st and 2nd tubes of series A. The contents of the 2nd tube are mixed, and 0.5 ml of this mixture is pipetted into the 3rd tube, mixed, and 0.5 ml of this mixture is pipetted into the 4th tube etc. In this manner the contents of the next tube are twice diluted each time. From bottle B the tubes of series B are filled and from bottle C the tubes of series C. By this procedure the dilution series of a serum to be titrated is obtained as indicated in Table 1.

TABLE 1.

Series of dilutions of a serum for determination of antitoxin titre by the direct haemagglutination method.

Series of tubes	Preparation for dilution of the 1st tube			Series of dilutions; concentration in negative logarithms											
	Serum		0.5% rabbit serum ml	tube no.											
	preceding dilution ml	in ml		1	2	3	4	5	6	7	8	9	10	11	12
A	1 : 10	1.75	0.45	1.1	1.4	1.7	2.0	2.3	2.6	2.9	3.2	3.5	3.8	4.1	4.4
B	1 : 10	1.35	0.79	1.2	1.5	1.8	2.1	2.4	2.7	3.0	3.3	3.6	3.9	4.2	4.5
C	1 : 10	0.95	0.94	1.3	1.6	1.9	2.2	2.5	2.8	3.1	3.4	3.7	4.0	4.3	4.6
				reciprocal values of dilutions											
A				12.5	25.1	50.1	100	200	398	794	1580	3160	6310	12500	25100
B				15.8	31.6	63.1	126	251	502	1000	2000	3980	7940	15800	31600
C				20.0	39.8	79.4	158	316	631	1260	2510	5010	10000	20000	39800

The same method of diluting is applied to a standard serum which contains, say, 10 AU/ml. After this, 0.05 ml of a suspension of sensitized blood cells is pipetted into each tube, and the contents are shaken for 30 seconds. Readings can be obtained after about 6 hours. The standard serum dilution which barely fails to agglutinate the added blood cells is chosen as end-point.

This end-point is as a rule constant if the blood cells are always prepared in the same way. The reaction in tube 10 of series C proved to meet this requirement even after the preparation of various charges of blood cells sensitized with diphtheria or tetanus toxoid. This tube contains 10AU : 10.000 = 0.001 AU standard serum.

The serum titre is calculated by multiplying by the factor 0.001 the reciprocal value of the dilution which gives the same picture as the standard in the haemagglutination series.

Concentrated sera often contain components which cause aspecific agglutination; the serum to be titrated must, therefore, be 10 × diluted in advance. This dilution in advance sufficiently reduces any possible disturbing influence.

The smallest quantity of antitoxin which can be determined by the direct haemagglutination method, is 0.0125 AU/ml serum.

Procedure of the haemagglutination inhibition test.

In determining the antitoxin titre of a serum by the haemagglutination inhibition test, titration can be effected at different levels, *e.g.*:

1. on the basis $L_A/10$, the quantity of test toxoid being equivalent to 0.1 AU standard serum.
2. on the basis $L_A/100$, the quantity of test toxoid being equivalent to 0.01 AU standard serum.
3. on the basis $L_A/1000$, the quantity of test toxoid being equivalent to 0.001 AU standard serum.

Since the procedure in determining tetanus and diphtheria antitoxins at different levels is the same in principle, it is sufficient to describe a tetanus titration on the basis $L_A/100$.

Determination of quantity of test toxoid on the basis $L_A/100$.

Quantities of standard serum, toxoid and 0.5% rabbit serum in saline solution, as indicated in the following scheme, are pipetted into a series of 12 tubes. All dilutions are made with 0.5% normal rabbit serum in saline solution (Table 2).

TABLE 2.

Tube No.	ml standard serum 0.02 AU/ml	ml toxoid		ml 0.5% normal rabbit serum
1	0.50	0.40	0.1 Lf per ml	0.10
2	0.50	0.36		0.14
3	0.50	0.32		0.18
4	0.50	0.28		0.22
5	0.50	0.24		0.26
6	0.50	0.20		0.30
7	0.50	0.32	0.05 Lf per ml	0.18
8	0.50	0.28		0.22
9	0.50	0.24		0.26
10	0.50	0.20		0.30
11	0.50	0.16		0.34
12	0.50	0.12		0.38

A one-hour interval is allowed after mixing, after which 0.05 ml of the sensitized blood cell suspension is pipetted into each tube. The tubes are shaken for 30 seconds and kept at room temperature for 16-20 hours, after which the reaction is read.

The end-point selected was the smallest quantity of tetanus toxoid which, when mixed with 0.01 AU standard serum, only just prevents agglutination of the added sensitized erythrocytes. In applying the above scheme it was found that the mixture in tube No. 6 met the requirements. The test dose of toxoid, therefore, is 0.20 ml 0.1 Lf/ml. After preparation of a new batch of sensitized erythrocytes the quantity of test toxoid should always be determined anew.

The quantity of test toxoid having been determined, the serum to be titrated is diluted according to the following scheme, and the dilutions are mixed with the test toxoid contained in the tubes and with 0.5% rabbit serum (Table 3).

In practice the error in this determination does not exceed 20% – hence the choice of dilution factor 1.2.

In preparing the control series the following dilutions were made:

1. tetanus standard serum 1 ml = 0.04 AU
2. tetanus standard serum 1 ml = 0.02 AU
3. tetanus toxoid 1 ml = 0.1 Lf.

After mixing a 1-hour interval is allowed; 0.05 ml of the suspension of sensitized erythrocytes is then pipetted into each tube, which is shaken for 30 seconds. The reaction is read after 16-20 hours' standing at room temperature. The end-point chosen is the serum

TABLE 3.

Titration scheme for haemagglutination inhibition test on the basis $L_A/100$.

Tube no.	number of AU/ml calculated	ml of 10 × diluted serum to be titrated		ml toxoid 0.1 Lf per ml	ml 0.5% rabbit serum
1	0.020	0.50	not further diluted	0.20	0.30
2	0.024	0.42		0.20	0.38
3	0.029	0.35		0.20	0.45
4	0.033	0.30		0.20	0.50
5	0.040	0.25		0.20	0.55
6	0.048	0.21		0.20	0.59
7	0.055	0.18		0.20	0.62
8	0.067	0.15		0.20	0.65
9	0.086	0.58	1+4 diluted	0.20	0.22
10	0.100	0.50		0.20	0.30
11	0.120	0.42		0.20	0.38
12	0.143	0.35		0.20	0.45
13	0.167	0.30		0.20	0.50
14	0.200	0.25		0.20	0.55
15	0.238	0.21		0.20	0.59
16	0.278	0.18		0.20	0.62
17	0.333	0.15		0.20	0.65
18	0.440	0.57	1+24 diluted	0.20	0.23
19	0.520	0.48		0.20	0.32
20	0.625	0.40		0.20	0.40
	Controls:				
1	0.006	0.30	0.02 AU/ml standard serum	0.20	0.50
2	0.007	0.35		0.20	0.45
3	0.008	0.42		0.20	0.38
4 [1]	0.010	0.50		0.20	0.30
5	0.012	0.60		0.20	0.20
6	0.014	0.35	0.04 AU/ml	0.20	0.45
7	0.016	0.42		0.20	0.38
8	0.020	0.50		0.20	0.30

[1]) tube No. 4 of the control series therefore contains 0.01 AU serum.

dilution, which when mixed with the quantity of test toxoid, just fails to cause agglutination of the cells. Since all sera have been diluted 10 × in advance, the serum titre values found must be multiplied by 10.

If a serum is titrated by the haemagglutination inhibition test just described, the smallest quantities of antitoxin which can be

TABLE 4.

Tetanus antitoxin titres expressed in AU/ml.

Serum No.	in-vitro method				in-vivo method		
	$L_A/10$	$L_A/100$	$L_A/1000$	direct method	$L_+/100$	$L_+/1000$	$L_+/10000$
1	0.86	0.77	3.12	1.58	1.10	1.20	1.20
2	0.86	1.20	2.50	2.00	1.10	0.60	0.35
3	4.40	1.20	2.50	1.26	1.10	1.20	1.20
4	<0.20	<0.20	<0.02	<0.012	<0.10	<0.01	0.003
5	4.80	8.60	7.70	12.50	4.50	6.40	4.80
6	23.80	30.50	77.00	50.10	24.00	32.00	48.00
7	4.80	3.30	3.40	5.01	4.50	4.80	4.80
8	<2.00	<0.20	0.012	<0.012	<0.10	<0.01	0.008
9	2.90	2.78	7.70	5.01	4.80	4.80	6.40
10	<2.00	<0.20	0.15	0.20	0.10	0.16	0.16
11	5.20	10.00	7.70	10.00	4.80	4.80	6.40
12	<0.20	<0.20	0.23	0.20	<0.10	0.12	0.32
13	0.40	0.67	0.77	0.40	0.60	0.32	0.16
14	12.00	18.60	34.20	25.10	19.20	19.20	25.60
15	<2.00	3.33	5.13	3.98	3.20	2.40	3.20
16	0.40	0.55	1.16	0.63	0.60	0.48	1.28
17	<2.00	<0.20	0.10	0.04	<0.10	<0.01	0.032
18	<2.00	<0.20	<0.02	<0.012	<0.10	<0.01	0.001
19	4.80	4.00	7.70	5.01	2.40	2.40	6.40
20	<2.00	<0.20	0.02	0.032	<0.10	0.04	0.192
21	<2.00	<0.20	0.10	0.10	<0.10	0.12	0.32
22	4.40	6.70	11.60	12.50	4.80	9.60	12.80
23	1.00	2.78	5.13	3.98	2.40	3.20	3.20
24	1.67	1.00	0.77	1.00	0.60	1.20	3.20
25	0.48	0.40	1.16	0.40	1.60	3.20	6.40
26	<2.00	0.067	0.068	0.079	<0.10	<0.01	0.004
27	0.33	0.27	0.10	0.063	<0.10	0.08	0.32
28	2.00	3.33	2.50	2.00	2.40	3.20	3.20

determined on the bases $L_A/10$, $L_A/100$, $L_A/1000$ are 2.0, 0.2 and 0.02 AU ml respectively.

Results.

Tables 4 and 5 show the antitoxin titres found in the available sera, as determined by the in-vivo and the in-vitro methods.

These tables show that the antitoxin titres obtained by the haemagglutination method agree fairly well with the in-vivo titres found. In practice the determinations $L_A/10$ and $L_A/100$ are less

TABLE 5.

Diphtheria antitoxin titres expressed in AU/ml.

Serum No.	in-vitro method				in-vivo method		
	$L_A/10$	$L_A/100$	$L_A/1000$	direct method	$L_r/100$	$L_r/1000$	$L_r/10000$
1	1.32	2.00	5.13	3.16	1.85	3.20	3.20
2	0.55	0.44	0.64	0.32	1.00	1.92	1.60
3	25.80	23.80	102.00	50.10	19.00	24.00	32.00
4	<0.20	<0.20	0.15	0.25	<0.10	0.10	0.13
5	<2.00	0.55	0.29	0.05	<0.10	<0.01	0.008
6	4.70	6.10	15.20	10.00	6.80	12.80	12.00
7	<2.00	<0.20	0.15	0.10	<0.10	0.06	0.032
8	<2.00	<0.20	0.068	<0.012	<0.10	<0.01	<0.001
9	1.20	1.00	2.85	0.80	0.96	1.60	1.20
10	<2.00	<0.20	<0.02	<0.012	<0.10	<0.01	<0.001
11	0.86	0.72	3.42	0.80	1.44	3.20	3.20
12	6.70	8.60	15.20	7.90	7.35	12.80	9.60
13	120.00	120.00	342.00	158.00	87.80	96.00	64.00
14	4.40	5.20	15.20	5.01	7.35	12.80	6.40
15	1.10	1.00	3.42	2.00	1.44	2.40	3.20
16	33.00	44.00	116.00	79.00	87.80	96.00	96.00
17	<2.00	<0.20	0.152	0.012	<0.10	<0.01	0.064
18	<2.00	<0.20	0.342	0.126	<0.10	0.12	0.12
19	<2.00	2.00	7.70	3.98	3.25	4.80	4.80
20	<2.00	<0.20	<0.02	0.012	<0.10	0.03	0.03
21	<2.00	<0.20	0.34	0.080	<0.10	0.06	0.04
22	1.00	1.00	2.28	0.80	1.44	2.40	1.60
23	0.44	0.48	2.28	0.80	0.64	1.20	1.20
24	4.80	2.80	5.13	1.58	1.73	2.40	2.40
25	0.20	0.22	0.77	0.50	0.19	0.24	0.24
26	8.60	10.00	15.20	10.00	7.35	12.80	9.60
27	4.80	4.80	11.60	7.94	3.90	4.80	4.80
28	0.52	0.55	1.00	0.80	0.29	0.48	0.32

TABLE 6.

Calculated correlation coefficients of titres found.

Tetanus titrations			Diphtheria titrations		
titration method haemag-glutination	titration in vivo-method	correlation coefficient	titration method haemag-glutination	titration in vivo-method	correlation coefficient
$L_A/1000$	$L_+/\ 1000$	0.94⁹	$L_A/1000$	$L_r/\ 1000$	0.97⁴
$L_A/1000$	$L_+/10000$	0.92²	$L_A/1000$	$L_r/10000$	0.97³
direct method	$L_+/\ 1000$	0.95⁷	direct method	$L_r/\ 1000$	0.97⁵
direct method	$L_+/10000$	0.90⁷	direct method	$L_r/10000$	0.97⁸

useful because low antitoxin titres cannot be determined on these bases.

Table 6 presents the correlation coefficients between haemagglutination titrations and in-vivo titrations calculated by the rank correlation method (Dickson and Massey, 1951).

The relatively high correlation coefficients given in table 6 indicate that the titration methods compared give a good average agreement of results.

The diphtheria titres found by the direct haemagglutination method and the haemagglutination inhibition test on the basis $L_A/1000$, plotted against the $L_r/1000$ and $L_r/10.000$ in-vivo values, are presented in Figs. 1 and 2.

The tetanus titres found by the direct haemagglutination method and the haemagglutination inhibition test on the basis $L_A/1000$, plotted against the $L_+/1000$ and $L_+/10.000$ in-vivo values, are presented in Figs. 3 and 4.

Discussion.

Before entering into a detailed discussion of the advantages and disadvantages of the titration methods described, a few remarks must be made on the nature and object of the antitoxin determinations.

The 'standard' for all in-vivo titrations is the International Standard Serum made available by the W. H. O. This standard (diphtheria or tetanus antitoxin) has by definition an avidity = 1.

In actual fact this means that a dilution ratio = 1 has been ascribed to these standard sera. By definition, thus a 'normal' avidity is ascribed to all sera behaving like these standard sera in titrations at various levels.

A behaviour which, in terms of avidity, deviates from that of these standard sera can be manifested only if a given serum is titrated at different levels (*e.g.* $L_+/10$, $L_+/100$, $L_+/1000$ $L_+/10.000$, or $L_r/10$, $L_r/100$, $L_r/1000$, $L_r/10.000$). This is only done, of course, if the investigator is specially interested in avidity. An investigation of this type (titrations at different levels), moreover, requires a relatively large material, which is not always available.

For normal routine determinations, therefore, a choice must be made, not only as to the method to be used but also as to the level at which the sera under investigation are to be titrated. In this con-

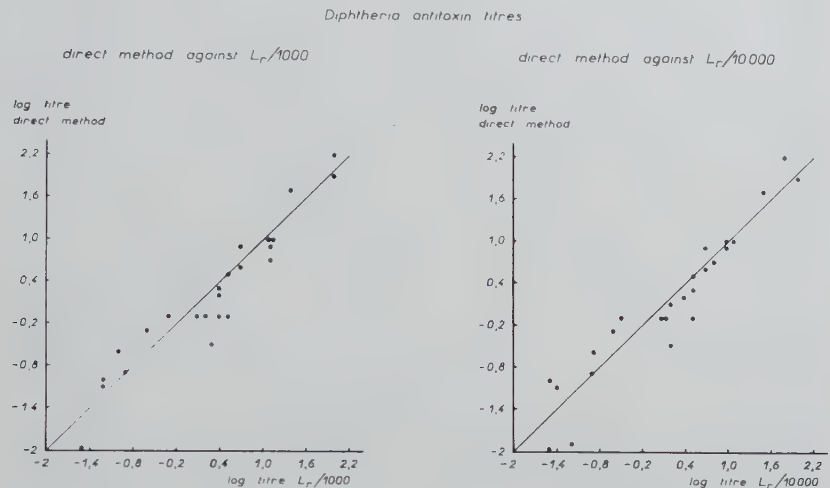

Fig. 1.

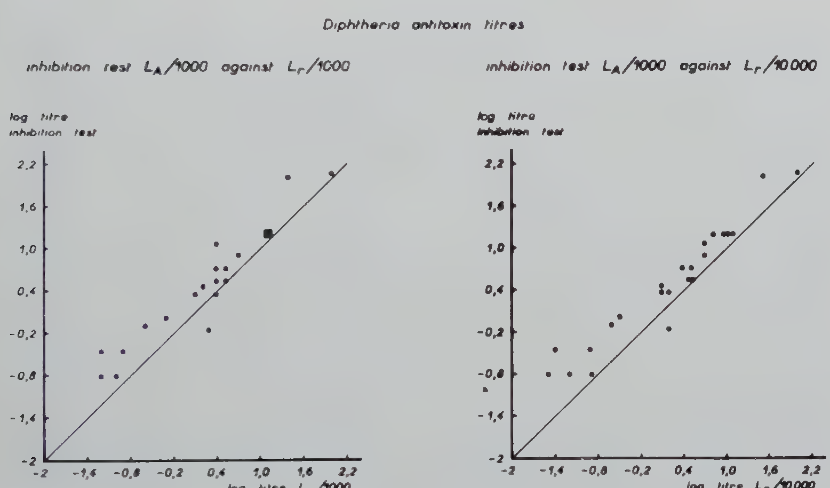

Fig. 2.

nection it should be realized that titration at a single level yields no information on the avidity of the serum concerned, and that the serum 'titre' obtained (expressed in AU/ml) has only a relative value. The 'titre' found at a given level is dependent to a considerable extent on the avidity of the serum investigated.

What are the factors on which the choice is based? Leaving the method used (in-vivo or in-vitro method) undiscussed for the time

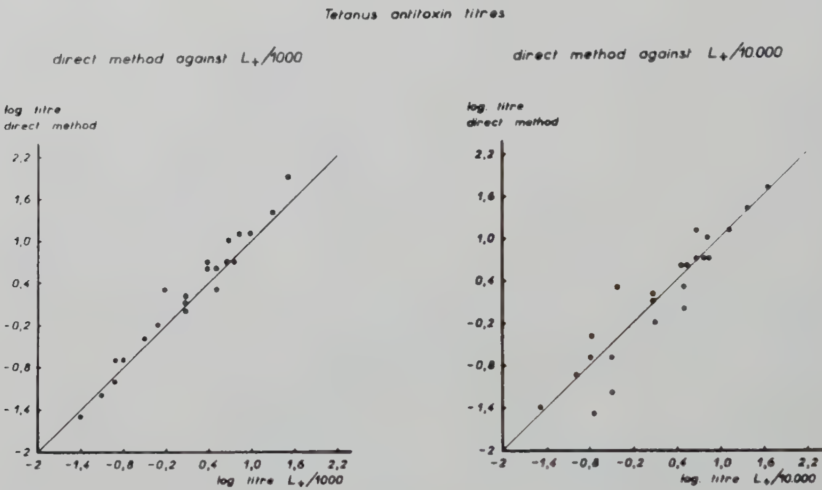

Fig. 3.

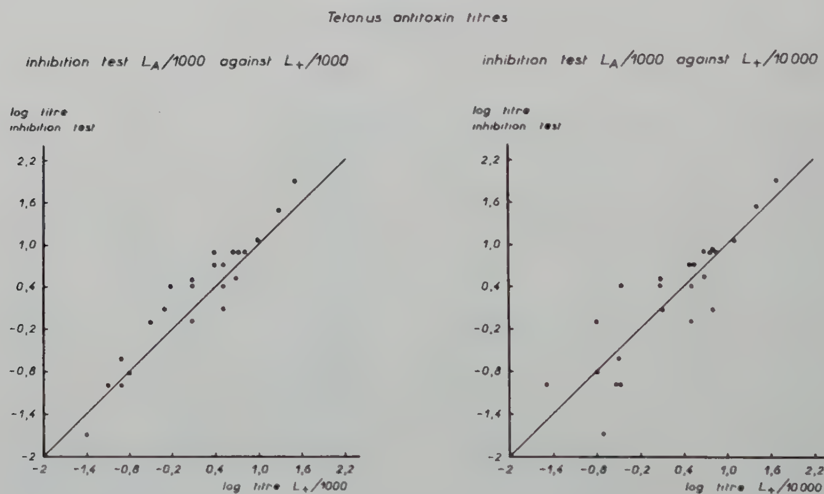

Fig. 4.

being, and confining ourselves to the level of titration, it can be stated that the choice is determined by the following factors:

1. The 'accuracy of determination' required, *i.e.* the degree of reproducibility of the results.
2. The serum titre to be expected, expressed in AU/ml.
3. The quantity of serum available.

The following considerations can be advanced with regard to the above three factors.

Apart from experimental errors in making up the serum and toxin mixtures (which can be reduced to very small dimensions by exact technique), the accuracy of titration to be attained is to a considerable degree dependent on the level at which titrations are made. According to our experience, the errors occurring are of the following order of magnitude:

$L_+/10$, $L_+/100$, $L_r/10$, $L_r/100$	about	5%
$L_+/1000$, $L_r/1000$,	,,	20%
$L_+/10.000$, $L_r/10.000$,	,,	100%
Direct haemagglutination method	,,	20%
Haemagglutination inhibition test $L_A/100$	,,	20%
Haemagglutination inhibition test $L_A/1000$	,,	50% [1]

As to the factors mentioned under 2 and 3, it is obvious that lower titre values can be determined at the 'low' levels ($L_+/10.000$, $L_r/10.000$, $L_A/10.000$) than at the 'high' levels. In general, moreover, a relatively small quantity of material is sufficient for titration at a low level.

The object of antitoxin determination can be defined as follows: In some cases it may be necessary to determine with considerable exactness the antitoxin titre in a number of unrelated serum samples, *e.g.* in antitoxic sera to be used in the treatment of human subjects (diphtheria and tetanus sera). These sera invariably have a relatively high antitoxin concentration (1000-5000 AU/ml), and amply sufficient material is always available for determination of these titres. Since an animal experiment is internationally required in this connection, these sera must be titrated in an in-vivo determination at a level of $L_r/10$ or $L_+/5$, or $L_r/100$.

The situation is entirely different in the titration of series of sera originating from human or animal groups, which per group, have received comparable treatment, *e.g.* series of patients immunized against diphtheria or tetanus in different but comparable ways. In these cases the absolute value of the titres obtained is as a rule of less importance than the interrelationship or proportion of the titres found per patient or per group of patients, regarded against the background of the prophylactic or therapeutic method used or the various stages during a period of observation. The titre values to

[1]) The International Pharmacopoea (1951) gives an error of about 11% for determination of tetanus antitoxin at the level of $L_+/5$, using 6 mice per titration, and one of about 5% for titration of diphtheria antitoxin at the level of $L_r/100$.

be expected, moreover, are often relatively low, while the quantity of serum available is generally small.

In such cases it is advisable to carry out all titrations relating to one patient or of several groups of comparable patients, at one level of titration.

In this connection, however, it must be borne in mind that the comparability of the titre values found per patient is only relative because the avidities of the individual serum samples per patient may change as time passes. No impression of this can be obtained by 'simple titration' (at only one titration level). This last objection could be overcome by carrying out at least two titrations per serum sample (*e.g.* at the levels $L_+/100$ and $L_+/10.000$).

It is obvious that – for practical reasons – this is but seldom practicable (or necessary).

Regarding the practical data of this paper against the background of these more or less theoretical considerations, we can sum up as follows:

From the results obtained, and in view of the correlation coefficients, it can be concluded that determination of diphtheria and tetanus antitoxins by the haemagglutination method is certainly useful for purposes of comparison. For practical purposes, we would prefer the direct method to the haemagglutination inhibition test, because the former determination can be made in a simple way without complex calculations. All sera with a diphtheria or tetanus antitoxin titre >0.012 AU/ml can be titrated in this manner. Determination on sera with a titre < 0.012 AU/ml should be repeated by an in-vivo titration method.

For this purpose titrations must be made at the levels of $L_+/10.000$ and $L_r/10.000$. Direct haemagglutination titration offers the following advantages:

1. no animal experiments
2. serum quantity required only 0.5 ml per titration
3. result of titration known as a rule on the same day, and certainly on the following morning.

The only disadvantage of the method lies in the fact that titres < 0.01 AU/ml cannot be determined in this way.

Summary.

A description is given of an investigation of the practical value of the determination of diphtheria and tetanus antitoxins by haemagglutination in sera from human sources.

A detailed discussion of the methods used is followed by a report on the value of these methods as tested on 28 sera obtained from volunteers in the National Institute of Public Health. The titres obtained by these in-vitro methods were compared with the results of animal experiments. Correlation coefficients were calculated for the values obtained at different comparable levels of titration. These coefficients proved to be very satisfactory.

General considerations regarding the nature and the object of such antitoxin determinations are presented and it is concluded that the haemagglutination technique described is useful in practice. This applies particularly to the titration of series of sera originating from one or several patients or from comparable groups of patients.

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SUPPORT FOR DIFFERENTIATION OF *ACETOBACTER* AND *ACETOMONAS*

by

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(Received June 1, 1960).

STOUTHAMER (1959) has recorded that amongst the 20 strains of acetic acid bacteria he studied, it was only in those of FRATEUR's (1950) 'suboxydans group' that the citric acid cycle was absent.

As the former 'suboxydans group' is synonymous with the later genus *Acetomonas* Leifson (1954), this evidence seemed to us to be further final and conclusive support for LEIFSON's differentiation of *Acetomonas* from *Acetobacter*. In STOUTHAMER's hands, however, one strain of the latter genus (FRATEUR's authentic one of *A. mesoxydans* var. *saccharovorans*) whilst possessing the citric acid cycle, did not oxidize ethanol further than acetic acid (a main negative character of *Acetomonas*). STOUTHAMER therefore concluded that this strain occupied a position intermediate between the mesoxydans and suboxydans groups; this is equivalent to saying that it was an intermediate between LEIFSON's amended *Acetobacter* genus and his new genus *Acetomonas*.

STOUTHAMER concluded that the behaviour of this one strain could be taken to 'stress the phylogenetic relations between the four *Acetobacter* groups (and the unity of the whole genus)'.

As, however, *Acetobacter* is a peritrichously flagellated genus whilst *Acetomonas* is a polar flagellated one; and as FRATEUR himself (1950) describes his *A. mesoxydans* var. *saccharovorans* as strongly 'over-oxidizing' ethanol through acetic acid to CO_2 and H_2O, we asked Dr. STOUTHAMER for a culture of his strain. This unfortunately had been lost, but Dr. STOUTHAMER told us (private communication; STOUTHAMER, 1960) that before this occurred he had retested it, and had been unable to repeat his published result.

He considered that the phenomenon might be an unstable one, akin to the mutations of other properties previously described by one of us (SHIMWELL 1959). By the courtesy of Prof. T. O. WIKÉN, however, we were able to obtain the identical strain from Delft.

We have found it to be as described by FRATEUR (1950) *i.e.* a very strong and rapid oxidizer of ethanol through acetic acid to CO_2 and H_2O. Thus, on 1% Difco yeast-extract/1.5% (w/v) ethanol/brom-cresol-green (pH range 5.4-3.8)/agar slopes, the indicator changed from blue to yellow and back to blue in three days at 27° C., indicating the oxidation of the ethanol to acetic acid (to yellow) and then further to CO_2 and H_2O (to blue again).

Similarly, on 1% YE/2% ethanol (w/v)/2% $CaCO_3$/ agar oxydogrammes (FRATEUR 1950), the $CaCO_3$ was rapidly dissolved in 2 days at 27° C., and was reprecipitated after a further 4 days, indicating oxidation of the calcium acetate formed to carbonate.

Thus *Acetobacter mesoxydans* var. *saccharovorans* is not an intermediate strain but a typical strongly over-oxidizing *Acetobacter* strain. STOUTHAMER's discovery of the presence of the citric acid cycle in *Acetobacter* and its absence in *Acetomonas* is thus of the greatest importance, not, we submit, in stressing the unity of the acetic acid bacteria as a single genus, but in fortifying LEIFSON's differentiation, for the position now is as follows:

(1) *Acetobacter* amended Leifson:
Ethanol completely oxidized through acetic acid to CO_2 and H_2O. Lactate and acetate oxidized to carbonate. If motile, flagellation peritrichous. Citric acid cycle present.
(2) *Acetomonas* Leifson (='suboxydans group'):
Ethanol only oxidized as far as acetic acid. Lactate and acetate not oxidized to carbonate. If motile, flagellation polar. Citric acid cycle absent.

As regards STOUTHAMER's findings that the strain concerned originally did not completely oxidize ethanol, we suggest that this was due to the use of washed suspensions or 'resting cells' by means of the Warburg technique, instead of normally growing cultures. For HROMATKA and EBNER (1948) have claimed (and we have fully confirmed) that centrifuging and washing acetic acid bacteria,

suspending them in buffer solutions etc. damages or interferes with their normal enzymic processes, the results then obtained being (nearly always quantitatively, and often qualitatively) quite different from those obtained with actively multiplying cells.

Acknowledgement.

We wish to thank Prof. J. Frateur for private communications expressing complete agreement with the conclusions reached in the above note, particularly the fortification of Leifson's differentiation.

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STUDIES ON A BETA-FRUCTOSIDASE (INULINASE) PRODUCED BY *SACCHAROMYCES FRAGILIS*

by

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(Received April 19, 1960).

Inulin is a naturally occurring plant polyfructoside. The linear chains consist of about 35 fructose molecules, united by β–1,2 linkages and terminating in a glucose molecule which is linked to fructose by an α-1,2 linkage as in sucrose (WHISTLER and SMART, 1953).

The ability of certain yeasts to ferment inulin was first recognized by LINDNER (1900), who showed that *Saccharomyces marxianus* and several unidentified yeasts vigorously fermented inulin. These observations were confirmed and extended by GRAFE and VOUK (1913) and by KLUYVER (1914). It is likely that KLUYVER worked with an imperfect strain of *Saccharomyces fragilis*. Much later it was definitely established by SACCHETTI (1933) that *S. fragilis* is able to ferment inulin. A few other species of yeast also have the ability to ferment inulin (see *e.g.* LODDER and KREGER-VAN RIJ, 1952).

Very little is known about the enzyme(s) catalyzing the hydrolysis of inulin to fermentable hexose sugars. WEIDENHAGEN (1941), in a review of the literature on "fructases", reported that baker's yeast autolysate contained a β-fructosidase which hydrolyzed inulin completely even though the intact yeast cells were unable to attack inulin. He believed that inulin was unable to penetrate the cell wall. ADAMS *et al.* (1943) demonstrated that highly purified invertase preparations, obtained from both baker's and brewer's yeasts, could

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hydrolyze inulin. Depending on the source of the enzyme and the method of purification, they found that the ratio of hydrolysis rates with sucrose and with inulin varied widely. Based on this and on different pH optima with sucrose and with inulin, they concluded that their preparations contained a separate enzyme, responsible for the hydrolysis of inulin, which was distinct from the β-fructosidase which hydrolyzes sucrose. The present work demonstrates that *S. fragilis* does not produce invertase, but a closely related, although distinct, inulinase. A description is given of the cultural conditions under which this yeast produces inulinase and of methods for the purification of the intracellular and extracellular enzyme.

Materials and Methods.

Substrates for growth. Two sources of commercially prepared inulin[1]) were used as a carbon source for growth and as a substrate for enzyme assays. No obvious differences were observed between the two brands.

Strains of yeast. Several strains of *S. fragilis* and *S. marxianus* were obtained from the yeast culture collection of the Department of Food Science and Technology at Davis. Most of the strains produced a full Durham vial of gas in 3 to 4 days at room temperature when a yeast autolysate medium containing 2 per cent inulin was used. No gas was produced by *S. cerevisiae*. *S. fragilis* strain 351 (which only ferments lactose after adaptation to this sugar) was used for most of the work, since this strain had been studied extensively in connection with its production of polygalacturonase (Phaff and Demain, 1956). Another strain (No. 106), which ferments lactose constitutively, also showed active fermentation of inulin and this strain was used in a few experiments. All starting inocula were grown for 24 hours on wort agar slants.

Determination of reaction rates. In a typical assay one ml of enzyme solution was added to 1.5 ml of the substrate solution in 0.1 M acetate buffer at pH 5.0 at 30° C. Two per cent solutions (final concentration) of sucrose or of inulin were used as substrates. The alkaline ferricyanide reagent of Schales and Schales (1945) was used to follow the increase in reducing groups

[1]) Mann Research Laboratories, Inc. New York, N.Y. and Nutritional Biochemicals Corp., Cleveland, Ohio.

in 0.1 ml samples which were removed periodically from the reaction mixture over a period of 15–30 minutes. With insoluble enzyme preparations larger volumes of reaction mixture were used. Samples were withdrawn and quickly centrifuged at high speed with a table model centrifuge. An aliquot was then withdrawn from the supernatant liquid for determination of reducing groups. One unit of hydrolytic activity (sucrose units or inulin units) was defined as that amount which releases 1 μmole of hexose (calculated as fructose from a standard curve prepared with this sugar) per minute at pH 5.0 and 30° C. For both soluble and insoluble enzyme preparations the enzymatic activity was shown to be proportional to the amount of enzyme used. The results were expressed as units of enzymatic activity per milliliter or per milligram protein for soluble preparations, and units of activity per mg of cells (dry weight) for insoluble preparations. The dry weight of washed cell suspensions was determined photometrically with a Klett-Summerson photoelectric colorimeter. A standard curve was prepared relating dry weight of cells to Klett units.

Enzyme preparations. Extracellular enzyme was prepared by centrifugation of the cells at high speed, followed by dialysis of the supernatant liquid against 0.1 M acetate buffer pH 5.0. Intracellular enzyme was prepared by washing the cells and resuspending them in 0.1 M acetate buffer pH 5.0. The suspension was then rapidly frozen and thawed (under tap water) five successive times. The final reaction mixture contained 0.03 M NaF to prevent fermentation of the hexose produced by enzymatic hydrolysis.

Protein determinations. Protein was determined by the method of Lowry *et al.* (1951) using crystallized bovine plasma albumin as the standard.

Paper chromatography. Analysis of the reaction products of enzymatic digests was done on Whatman paper No. 4 with n-propanol, ethyl acetate, and water (7 : 1 : 2) as the developing solvent. Reducing sugars were detected with the benzidine trichloroacetic acid spray of Bacon and Edelman (1951), or with the silver nitrate spray of Trevelyan *et al.* (1950).

Results.

Exploratory experiments. A preliminary test was made to determine if an inulin-hydrolyzing enzyme could be detected

in the culture liquid and (or) in the cells. *S. fragilis* was grown for 4 days at 23–25° C. without agitation in yeast autolysate with 2 per cent inulin. After separating the cells and culture fluid by centrifugation it was found that both fractions exhibited distinct enzymatic activity with inulin as the substrate. Sucrose and raffinose were also hydrolyzed by both preparations and at a higher rate than inulin.

Comparison with invertase. The hydrolytic properties, demonstrated above, gave no indication whether *S. fragilis* produced a characteristic inulinase or an invertase similar to that produced by baker's or brewer's yeast. In order to obtain evidence to support one or the other possibility, the ratio of the hydrolysis rates at pH 5.0 with sucrose and with inulin (hereafter referred to as the S/I ratio) were determined for the extracellular enzyme of *S. fragilis* and for a commercial invertase[1]) preparation. The S/I ratio for invertase was found to be about 14,000, while the ratio for the enzyme of *S. fragilis* was about 25. Although the difference between the preparations was very striking, the possibility was considered that the invertase preparation contained some α-glucosidase, which could contribute to the hydrolysis rate of sucrose. This can be avoided if raffinose is used as the substrate, in which the α-glucosidic bond is not located terminally. Consequently, invertase and the β-fructosidase of *S. fragilis* were assayed with raffinose and inulin at pH 5.0. The R/I ratio was 5,800 for invertase and 3.5 for the enzyme of *S. fragilis*. Thus, the very large differences in the above ratios for the two enzyme preparations indicated that they were dissimilar and it justified the name inulinase for the β-fructosidase of *S. fragilis*.

Measurement of intracellular inulinase. It was of interest to determine how much of the total enzyme activity associated with the cells was measured with inulin or sucrose as substrates and whether the cell-bound enzyme could be readily solubilized. Cells were subjected to freezing and thawing, or autolysis, and assays were made of the total enzyme activity of a treated suspension and of the activity in soluble form after centrifugation of the insoluble residue. Autolysis was done by adding 1 ml of chloroform and 1 ml of toluene to 20 ml of a cell suspension at 30° C. (about 100 mg/ml). The cells were agitated periodically.

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[1]) Technical grade. Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 1.
Effect of freezing and thawing or autolysis on the activity and solubility of intracellular inulinase.

Procedure	Total activity[a] units/mg cells with sucrose	Soluble activity[b] units/mg cells with sucrose
untreated[c]	9.9	—
frozen & thawed 1 ×	9.9	0.17
frozen & thawed 2 ×	9.3	0.27
frozen & thawed 4 ×	9.1	0.36
frozen & thawed 6 ×	10.5	0.60
frozen & thawed 8 ×	10.6	0.71
autolysis 24 hr	9.1	9.0
autolysis 44 hr	9.4	10.0

a) total activity refers to assays conducted with the cell suspension after a given treatment, but prior to centrifugation.
b) soluble activity refers to enzyme activity in solution after centrifugation at 8,000 × G for 30 minutes.
c) the untreated cells consisted of a suspension of inulin grown cells (97.5 mg cells/ml) in 0.1 M acetate buffer at pH 5.

The results in Table 1 show that the untreated cells exhibited full activity with sucrose, since it did not increase after the various treatments. Freezing and thawing solubilized only an insignificant fraction of the cell-bound enzyme, whereas autolysis proved to be an effective procedure to solubilize the enzyme quantitatively. When similar assays were done with inulin, it was found that the activity of untreated cells could be increased three- to four-fold by freezing and thawing five times. Additional treatments did not increase the total activity significantly. Autolysis (which solubilizes all the enzyme) approximately doubled the activity as compared to the frozen and thawed preparation (or about a six-fold increase of the original cells). Thus, the S/I ratio of untreated cells was about 140 and decreased to about 40 after freezing 5 times, and to 20–25 in autolyzed cells. The last figure is the same as that for the extracellular enzyme. Presumably the contact between inulin and cellbound enzyme is facilitated by freezing and thawing, but maximal activity is obtained only after autolysis. Since in many cases assays were made with inulin as well as with sucrose, cell suspensions were always frozen and thawed 5 times prior to an assay.

Effect of strain. In order to see if the above results also applied to a strain of *S. fragilis* which had a constitutive β-galactosidase (see Methods and Materials), strain No. 106 was grown with

inulin on a shaker. The S/I ratio of the culture liquid was 25 and that of the cells (frozen and thawed 5 times) was 56. This indicated that this strain, too, did not produce invertase, but only inulinase.

Reaction products of inulin hydrolysis. When inulin was treated with an enzyme preparation and the reaction products were periodically analyzed by paper chromatography, fructose was the only reaction product detectable, except that towards the end of the reaction small amounts of glucose also appeared on the paper. When α-methyl-D-glucoside or melezitose were treated with the enzyme preparation, no increase in reducing value could be detected by the method of Schales and Schales (1945). This demonstrated the absence of an α-glucosidase in our preparation, and it was concluded that the enzyme acted in an endwise fashion, sequentially hydrolyzing fructose molecules from the end of the chain opposite to that carrying the terminal glucose residue.

Enzyme production in different media. First it was shown that with inulin much greater yields of enzyme could be obtained in aerated cultures (on a rotary shaker or by sparging with air) than in standing cultures. The increased amount of enzyme was due primarily to higher cell yields rather than to a specific stimulation of inulinase production.

Thus far yeast autolysate was used as a source of nitrogen for the growth of the yeast. Since much purer extracellular enzyme solutions might be expected in the absence of protein in the culture medium, other sources of nitrogen were tried. Yeast nitrogen base (Difco), in which $(NH_4)_2SO_4$ is the principal nitrogen source, gave poor yields of cells, probably because the pH during growth dropped rapidly from about 5.0 to 2.4 or less. At such low pH values inulinase has little activity on inulin – the substrate for growth. A synthetic medium was therefore devised to which various nitrogen sources could be added. This basal medium, used for all subsequent experiments, had the following composition (all components in unit weight per liter of distilled water):

Salts (g)		Vitamins (μg)		Trace elements (μg)	
KH_2PO_4	1	biotin	2.5	$Fe(NH_4)_2(SO_4)_2$	14,000
$MgSO_4.7H_2O$	0.5	Ca-pantothenate	400	$Zn\ SO_4.2H_2O$	3,000
NaCl	0.1	inositol	2000	$MnSO_4.4H_2O$	2,000
$CaCl_2$	0.1	niacin	400	$CuSO_4.5H_2O$	300
		p-aminobenzoic acid	200	$(NH_4)_6Mo_7O_{24}.4H_2O$	90
		pyridoxine . HCl	400	$Na\ VO_3.4H_2O$	40
		thiamine . HCl	400	H_3BO_3	570

When Casamino acids (acid hydrolyzed casein) were used as the nitrogen source good growth and enzyme production were obtained. However, not all of the nitrogenous components of casamino acids were dialyzable. Next, ammonium phosphate was tested as the nitrogen source. With 0.5 percent diammonium phosphate the initial pH of the medium was 7.1–7.2 and growth was very latent due to the low activity of inulinase at this pH. With 1.2 per cent $NH_4H_2PO_4$ and 1 per cent inulin the initial pH was 4.5. Growth and enzyme production were satisfactory, but the pH slowly dropped to 2.6 during the first 24 hours of growth. As there was a possibility that some of the extracellular enzyme might become inactivated by exposure to low pH values over a period of several days, succinate buffer at pH 5.0 (final concentration 0.025 M) was added to the medium containing 1.2% $NH_4H_2PO_4$ and 0.5% inulin. After 24 hours the pH changed from 5.1 to 5.8. The small rise in pH was probably due to the utilization of some of the succinate ion by the yeast. Raising the inulin concentration from 0.5 to 3 per cent showed that much better cell yields and enzyme yields were obtained at a level of 1 per cent. Above this concentration, however, the somewhat higher yields did not justify the use of larger amounts of substrate.

A comparison was also made of autoclaving *versus* Seitz filtration of the inulin substrate. No profound differences in enzyme production were noted, although the lag period of growth was generally shorter in autoclaved media.

Replacing yeast autolysate with ammonium phosphate as the nitrogen source raised the specific activity of the dialyzed culture medium from 0.6 to a range of 5–8 inulin units per mg protein. The cell yields after 24 hours of growth on a rotary shaker were generally between 3 and 5 mg (dry weight basis) per ml. It was also shown in these preliminary experiments that per unit volume of culture medium the cells contained two to three times as much inulinase as the culture fluid. The latter usually contained from 4–12 sucrose units per ml and the cells 6–30 sucrose units per ml.

Effect of carbon source. *S. fragilis* was grown with various carbon sources and the amount of extracellular enzyme produced is shown in Table 2.

Inulinase appears to be an inducible enzyme. These experiments were repeated a number of times. Although the results varied somewhat, the highest yield of enzyme was always obtained with

TABLE 2.

Effect of carbon source on extracellular inulinase production. Basal medium plus 1% casamino acids. Rotary shaker (250 rpm) at 20° C. for 48 hours.

Carbon source (2%)	Inulin units/ml
inulin	0.3
raffinose	0.15
sucrose	0.03
galactose	0.05
glucose	0.05
fructose	0.1
mannitol[a])	0.05
sorbitol[a])	0.1

[a]) grown for 66 hours.

inulin. Raffinose was a fairly good inducer. With sucrose, however, very low enzyme yields were obtained. The S/I ratio of the enzyme produced with sucrose was 28, which is very similar to the figures obtained with inulin as the carbon source.

Fermentation of inulin. Cells grown on a shaker with glucose, sucrose, or inulin as carbon sources were found to ferment their respective substrates in a Warburg respirometer with $Q_{CO_2}^{N_2}$ values of 40–50. Thus, inulin-adapted cells can ferment this substrate at the same rate as glucose. On the other hand, glucose- or sucrose-grown cells fermented inulin only after a lag of about 3½ hours, and the maximum rate, achieved after 7 hours, was less than half of the $Q_{CO_2}^{N_2}$ values given above. It can be calculated that the amount of "active" inulinase in inulin-grown intact cells is sufficient to account for a fermentation rate equal to that of glucose. Taking one of the lowest values obtained for the amount of inulinase in inulin-grown cells (0.1 inulin unit/mg of cells) and knowing that about 1/4 of this amount is active in intact cells, this amount of enzyme can produce 0.025 μmoles of fructose per minute per mg of yeast. This corresponds to 1.5 μmoles per hour or 3 μmoles of CO_2 per hour per mg, which is more than sufficient for a $Q_{CO_2}^{N_2}$ value of 50. The presence of extracellular inulinase is therefore not essential for maximum fermentation rates.

Purification and comparison of extra- and intracellular inulinase.

Before studying the properties of the enzyme in more detail it

was necessary to isolate and purify the intra- and extracellular enzyme. Demonstration of the identity of the intra- and extracellular enzyme would be a requisite for studies dealing with enzyme excretion (to be reported in a later paper).

Four liters of basal medium containing 1 per cent inulin, 1.2 per cent $NH_4H_2PO_4$ and 0.025 M succinate were inoculated with adapted cells and sparged with sterile air for 48 hours at 23-25° C. Dow antifoam A was added to minimize foaming. After centrifugation, the supernatant liquid was allowed to stand for 24 hours to allow breakdown of a possible residue of non-utilized inulin. This can also be accomplished by dialysis against acetate buffer at pH 5.0 for 24 hours.

Extracellular enzyme. Both calcium phosphate gel and diethyl-amino-ethyl (DEAE) cellulose[1]) were tried as adsorbents. The latter adsorbent was developed by PETERSON and SOBER (1956). DEAE cellulose was pretreated by suspending it several times in distilled water and decanting all fine particles that did not settle in 20 minutes. The remainder was washed with 0.01 M acetate buffer at pH 5.0 until the pH remained constant at 5.0. Direct adsorption from the centrifuged culture liquid was ineffective, but after dialysis against 0.01 M acetate buffer at pH 5.0 or against tap water (pH about 7.5) both adsorbents were effective. The enzyme could be recovered from the phosphate gel by treating it with 1 M acetate buffer at pH 5.0. Elution from the DEAE cellulose was accomplished with 0.1 M acetate buffer at pH 5.0.

DEAE cellulose was chosen for further work. Precipitation of the eluate, representing a concentrated inulinase preparation, was attempted with ammonium sulfate. However, neither at pH 5.0 nor at pH 3.0 did a precipitate form in saturated $(NH_4)_2SO_4$ at 0° C. Also, addition of trichloroacetic acid in various concentrations to the concentrated enzyme solution failed to form a precipitate. It was possible, however, to precipitate the enzyme with acetone (30 per cent v/v). The acetone was precooled to -10° C. and added slowly to the enzyme solution at 0° C. The precipitate was centrifuged at 8000 $\times$ G for 30 minutes in the cold and the precipitate was dissolved in 0.1 M acetate buffer at pH 5.0, dialyzed to remove the last traces of acetone and stored at 5° C. Such a preparation lost no

[1]) Type 40; reagent grade; capacity 0.88 meq/g; Brown Co., Berlin, New Hampshire.

TABLE 3.

Summary of extracellular and intracellular inulinase concentration and purification.

Extracellular fraction	Volume ml	units/ml with sucrose (S)	units/ml with inulin (I)	S/I	mg protein/ml	units/mg protein with sucrose	total units with sucrose
cell free							
culture fluid	3,410	24	1.05	23	0.110	219	82,000
dialysate[a])	3,630	21.5	0.625	34.5	0.034	630	78,000
absorbed							
culture fluid[b])	3,600	3.5			0.012	292	12,600
1st eluate[c])	22.6	1,350	52.5	26	1.800	750	30,500
2nd eluate	21.6	550	17.5	31.5	0.800	690	11,900
3rd eluate	23.0	143	6.25	23	0.290	490	3,290
4th eluate[d])	24.0						1,260
acetone precipitate of 1st & 2nd eluates[e])	5.0	4,100	205.0	20	6.100	670	20,500
Intracellular fraction							
autolysate supernate[f])	60	1,700	77.5	22	30.4	56	102,000
ammonium sulfate							
supernate[g])	69	1,225	42.5	26.5	20.7	54	78,000
dialysate[h])	119	790	24.5	32	1.8	440	94,000
acetone precipitate[i])	15	3,550	175	20	6.1	580	53,000

[a]) dialyzed for 48 hours against running tap water (pH 7.5) at room temperature.

[b]) culture fluid after adsorption overnight with 5 g DEAE cellulose followed by separation of the adsorbent.

[c]) DEAE cellulose collected by filtration, resuspended in 25 ml 0.1 M acetate buffer at pH 5 for 30 minutes and filtered.

[d]) DEA cellulose eluted with 25 ml 0.1 M acetate buffer at pH 4.

[e]) eluates 1 and 2 combined, precipitated with acetone at 30 per cent (v/v) at 0° C., precipitate collected by centrifugation and dissolved in 0.01 M acetate buffer at pH 5.

[f]) autolysis of about 20 g (dry weight) cells, collected by centrifugation, suspended in 50 ml 0.1 M acetate buffer pH 5, 1 ml chloroform and 1 ml toluene, autolyzed at 30° C. for 16 hours and centrifuged at 8,000 G for 30 minutes.

[g]) precipitated with saturated ammonium sulfate (30 minutes in an ice bath) and centrifuged at 8,000 G for 30 minutes.

[h]) dialyzed against 0.01 M acetate buffer at pH 5.

[i]) acetone precipitation carried out as under (e) above and precipitate dissolved in 15 ml 0.01 M acetate buffer pH 5.

activity when stored for 4 months. Table 3 shows the results of such a concentration and purification procedure.

Intracellular enzyme. It was shown in Table 1 that autolysis for 24 hours was the best and most convenient procedure to solubilize the enzyme. Since inulinase appeared to be soluble in saturated $(NH_4)_2SO_4$, use was made of this property to precipitate foreign proteins in the autolysate at 0° C. with $(NH_4)_2SO_4$ at saturation. The salt was added slowly to the autolysate while stirring it. When saturation was reached the mixture was allowed to stand for 30 minutes and was then centrifuged for 30 minutes at 8000 × G in the cold. The supernatant liquid was dialyzed against 0.01 M acetate buffer at pH 5.0 until free of sulfate ions. Following this the solution was precipitated with acetone as was described for the extracellular enzyme. The results are presented in Table 3.

Comparison of the intra- and extracellular enzyme preparations. The ratios of hydrolysis rates with sucrose and with inulin (S/I ratios) were determined for the various fractions during purification. The results in Table 3 indicate that these ratios for the intra- and extracellular preparations were very similar, and ranged from 20–34.5. Since invertase from baker's yeast gave an S/I ratio of about 14,000 under the same assay conditions, *S. fragilis* probably does not produce even traces of an invertase as is found in baker's or brewer's yeast, since its presence would undoubtedly increase the S/I ratio significantly. Furthermore, the two purified preparations had about the same specific activity with sucrose.

Next, the heat sensitivities of the two preparations were compared.

TABLE 4.

Comparison of the effect of heating at 60°C. and pH 5.0 on purified preparations of intra- and extracellular inulinase..

Enzyme preparation	minutes exposure	units/ml with sucrose	units/ml with inulin	S/I
extracellular	0	11.0	0.50	22
,,	5	8.5	0.35	24
,,	10	6.0	0.275	22
,,	20	3.8	0.175	21.5
intracellular	0	12.0	0.50	24
,,	5	10.0	0.45	22
,,	10	6.75	0.30	22.5
,,	20	5.0	0.20	25

Preliminary tests showed that inulinase was reasonably stable at 50° C. and was rapidly destroyed at 70° C. Therefore 60° C. was chosen as the temperature for inactivation by heat of the purified enzymes. The data on heat inactivation, shown in Table 4, indicate that with both preparations the enzymatic activities with sucrose and with inulin were destroyed at the same rates, since the S/I ratios remained essentially constant. Rates of the inactivation for extra- and intracellular enzyme are plotted in figure 1. These data show that the two preparations behaved very similarly.

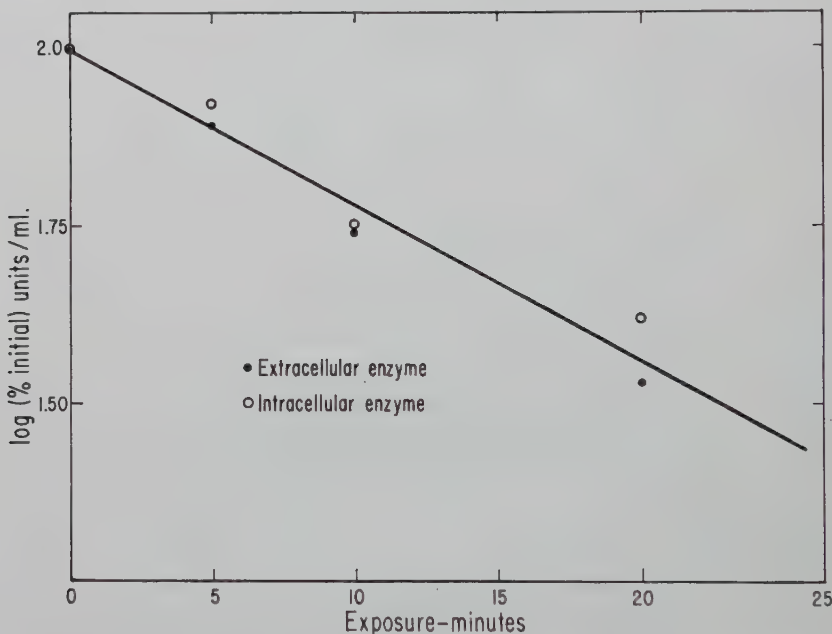

Fig. 1. Comparison of heat inactivation of extracellular and of intracellular inulinase preparations at 60° C. Data from sucrose assays.

The pH optima of the two enzyme preparations with sucrose and with inulin are shown in figures 2 and 3. Both the intra- and the extracellular enzyme preparations showed an optimum of 4.1–4.3 with sucrose and an optimum of 5.0–5.2 with inulin as the substrate.

Finally the intra- and extracellular preparations were compared with respect to their elution patterns from a column of DEAE cellulose. The adsorbent was prepared in the same manner as described in the section dealing with purification of the enzyme. A concentrated enzyme solution (prepared by acetone precipitation)

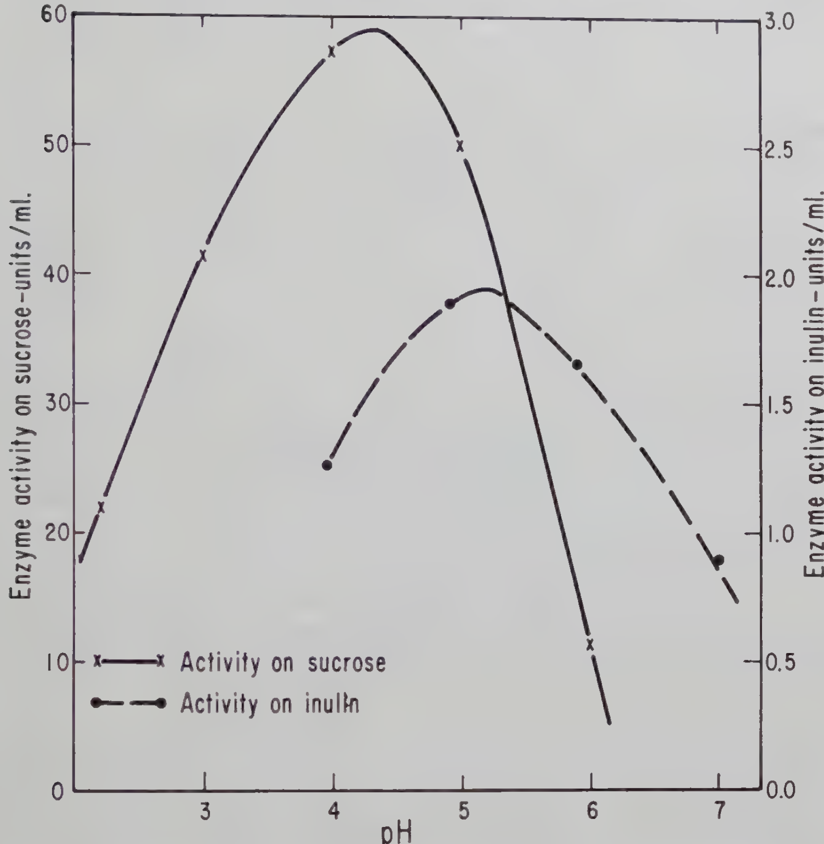

Fig. 2. pH optima of extracellular inulinase with sucrose and inulin as substrates.

was applied to the top of the column (17 cm long and 1 cm diameter) and gradient elution was done by applying NaCl – water mixtures of increasing concentration. Five milliliter fractions were collected with an automatic fraction collector. Figures 4 and 5 show the gradient elution patterns of the extra- and intracellular enzyme preparations, and figure 6 shows the elution pattern when equal portions of the two preparations were combined. The data show that in all cases a single protein peak appeared (expressed as the optical density at 280 mμ) at about 0.7 per cent NaCl. The open circles represent the activity with sucrose as the substrate and it can be seen that these activities follow the protein curve exactly. The figures accompanying the open circles represent the S/I ratios of

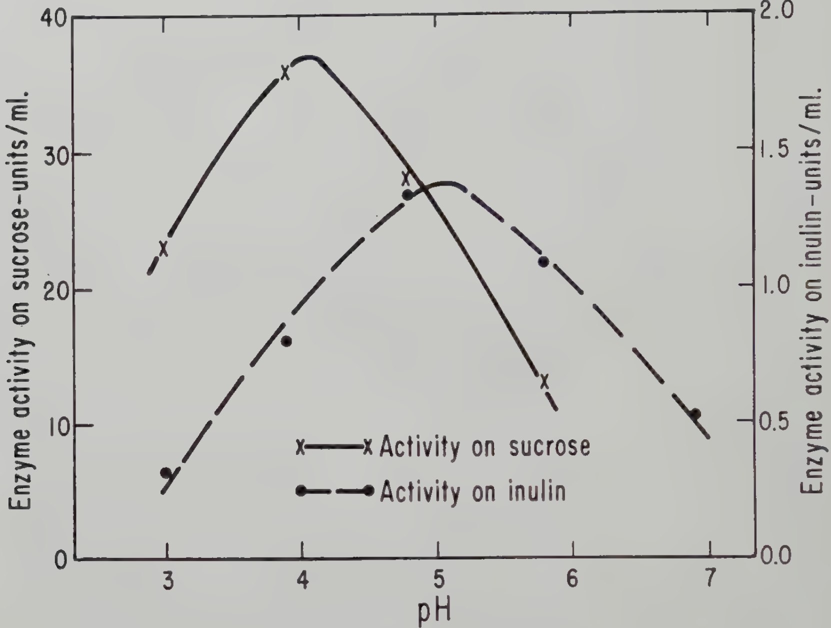

Fig. 3. pH optima of intracellular inulinase with sucrose and inulin as substrates.

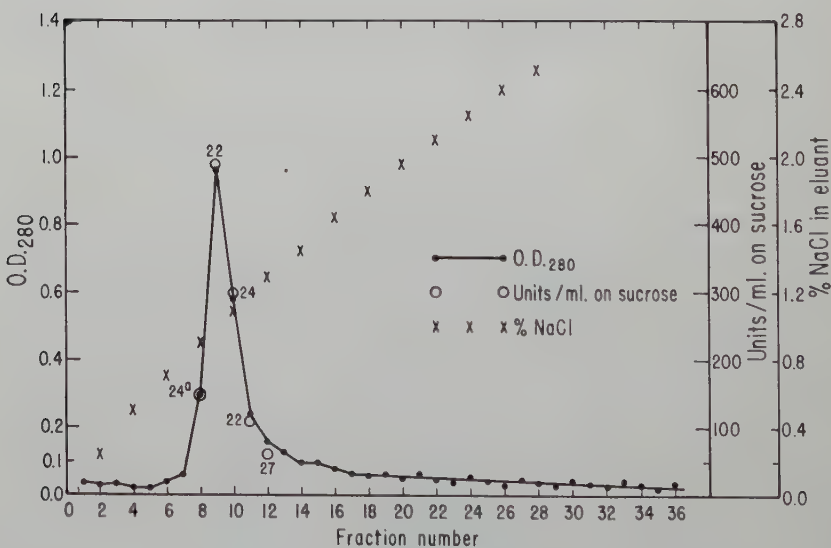

Fig. 4. Elution pattern of extracellular inulinase from a DEAE cellulose column.[a] The numbers indicate the S/I ratio of that fraction.

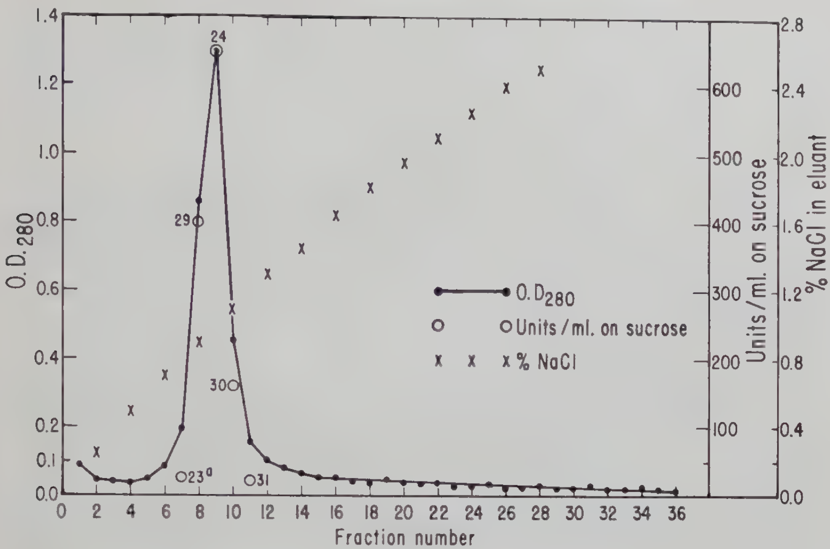

Fig. 5. Elution pattern of intracellular inulinase from a DEAE cellulose column.[a] The numbers indicate the S/I ratio of that fraction.

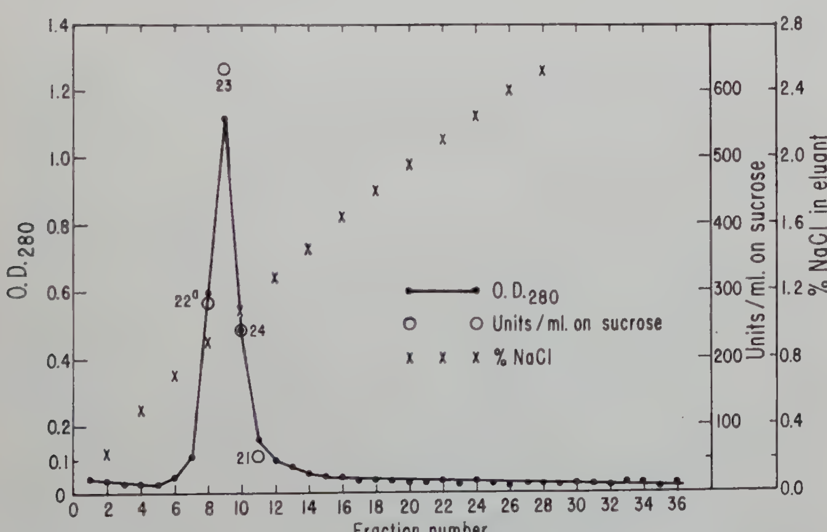

Fig. 6. Elution pattern of a mixture of extracellular and intracellular inulinase from a DEAE cellulose column.[a] The numbers indicate the S/I ratio of that fraction.

these samples. The constancy of these figures shows that both the activity with sucrose and that with inulin follow the protein curve. It may be concluded from these experiments that the intra- and the extracellular enzyme preparations are of high purity and that they are very similar or identical.

Substrate specificity. Besides inulin and sucrose, the inulinase of *S. fragilis* was found to hydrolyze raffinose and bacterial levans. Analysis by paper chromatography showed that raffinose was hydrolyzed to fructose and melibiose. Samples of bacterial levans produced by *Aerobacter levanicum* (Hestrin *et al.*, 1956) and by a species of *Corynebacterium* (Avigad and Feingold, 1957) were kindly supplied by Dr. G. Avigad. Our enzyme rapidly hydrolyzed both types of levan and produced fructose as the reaction product. The linkage of the straight chain sections in these levans of high molecular weight is β-2→6 and the branch points constitute β-2→1 linkages. These results show that inulinase can hydrolyze β-2,6 bonds linking two fructose units. Since the hydrolysis of β-2,1 bonds was previously shown to occur during the hydrolysis of inulin, it must be concluded that the levans were completely hydrolyzed by inulinase.

Discussion.

The investigations reported above have shown that *Saccharomyces fragilis* synthesized an inducible inulinase, which can hydrolyze inulin, sucrose, the fructose portion of raffinose, and bacterial levans. This enzyme differs from invertase (yeast sucrase) by its much more rapid action on inulin or levans. As a result, the ratio of hydrolysis rates of sucrose and inulin is much lower for inulinase (20 to 35 in the various experiments) than for invertase of baker's yeast (about 14,000). With regard to the absolute magnitude of the S/I ratios, it should be emphasized that these ratios are arbitrary and, for the following reasons, do not reflect the true relative rates of activity with the two substrates. First, no correction was made for the fact that during sucrose hydrolysis two reducing sugars are produced, whereas during cleavage of terminal fructose units from inulin only a single reducing group is exposed. Secondly, the concentration of both substrates was two per cent, which means an approximate fifteen fold difference in molar concentration. Although the enzyme was fully saturated by 2%

sucrose under our experimental conditions, this was not entirely the case with 2% inulin. The use of higher concentrations of inulin was not practical, because of the poor solubility of this polysaccharide. Thirdly, all assays were made at pH 5.0, the optimum pH of inulin hydrolysis, but not that of sucrose (pH 4.1–4.3). When the reaction pH is on the steep part of the pH- activity curve, small pH variations in different assays cause relatively large changes in reaction rates. This last factor is probably mostly responsible for the observed fluctuations in S/I ratios between 20 and 35. A correction for the first two factors would lower the S/I ratio and a correction for the pH factor would raise it. In spite of the relative nature of the S/I ratio, it is obvious that the presence of even a small fraction of true "invertase" would increase the S/I ratio appreciably. DEDONDER (1952) prepared a partially purified inulinase from the fungus *Sterigmatocystis nigra* and reported an S/I ratio of 5.5, based on an assay technique different from the one used by us. He also believes that a single enzyme is responsible for the hydrolysis of inulin and sucrose. His enzyme, which also splits off terminal fructose units, was precipitable by $(NH_4)_2SO_4$ and appears, therefore, to be different from the one studied here. ADAMS *et al.* (1943) reported S/I ratios for highly purified invertase preparations from baker's and brewer's yeast in the range between 2,800 and 28,300, based on methods entirely different from ours. The various figures, however, clearly demonstrate a large difference in S/I ratio for inulinase and invertase. This difference is shown even more clearly by the almost total lack of activity of invertase on bacterial levans with molecular weights between 10 and 20 million (AVINERI-SHAPIRO and HESTRIN, 1945; FEINGOLD, AVIGAD and HESTRIN, 1956) which are readily hydrolyzed by inulinase.

Another difference between invertase and inulinase is their optimum pH with sucrose and inulin. Our enzyme has an optimum pH of 4.3 with sucrose and 5.1 with inulin. ADAMS *et al.* (1943) reported an optimum pH of their invertase of 4.8–5.6 for sucrose, whereas maximum activity with inulin was found to be between pH 3.2 – 3.8. In fact, ADAMS *et al.* used these different pH optima as an argument for postulating a separate inulinase component in baker's yeast invertase. However, our pH data with inulinase and the results of DEMAIN and PHAFF (1954) and PHAFF and DEMAIN (1956) with pure yeast endopolygalacturonase have shown that polysaccharidases do not necessarily have the same optimum

pH for the hydrolysis of polysaccharides as for oligosaccharides.

Finally, invertase and inulinase behave differently with respect to their induction. The literature contains numerous more or less well supported observations that invertase of *Saccharomyces cerevisiae* is a constitutive enzyme, although its concentration can be increased by sucrose in the medium (*cf.* NEUBERG and ROBERTS, 1946). A recent paper by DWORSCHACK and WICKERHAM (1958) shows that invertase excretion by growing cells of *Saccharomyces uvarum* is markedly stimulated by sucrose. Inulinase of *S. fragilis*, on the other hand, is an inducible enzyme, the formation of which is not stimulated by sucrose, moderately well by raffinose and best by inulin.

The data of R. DAVIES (1953) – with another strain of *S. fragilis* – show that glucose and fructose strongly inhibit "invertase" synthesis, that sucrose is a poor inducer and that high levels of "invertase" are produced with raffinose. A. DAVIES (1956) noted marked inhibition by glucose in concentrations greater than 0.001% with continuous culture technique. Unfortunately, neither of these authors used inulin as a substrate. The fact that we found no invertase in two strains of *S. fragilis* and the similar results on induction of the Davies strain and our strain make it possible that the English workers actually may have studied inulinase rather than invertase.

Our results have also brought out more clearly the fact that yeasts which ferment sucrose do not necessarily contain the classical invertase. Sucrose fermentation (or assimilation) can be brought about with the participation of certain α-glucosidases, such as the one controlled by the M_1 gene in *Saccharomyces italicus* (WINGE and ROBERTS, 1950), by invertase (as in *S. cerevisiae*) and by inulinase (as in *S. fragilis*). The α-glucosidases, in contrast to the other two enzymes, do not attack raffinose.

Summary.

A study was made of a β-fructosidase, which is produced extracellularly and intracellularly by *Saccharomyces fragilis*. The enzyme catalyzes the hydrolysis of inulin, bacterial levans, sucrose, and the fructose portion of raffinose, by splitting off terminal fructosyl units. It attacks β-2,1 as well as β-2,6 linkages. The enzyme content of inulin-grown cells is sufficient to allow fermentation of inulin at the same rate as glucose. The ratio of hydrolysis rates with sucrose and

inulin was about 25 for the β-fructosidase of *S. fragilis* and about 14,000 for invertase. *S. fragilis* does not contain significant amounts of invertase and it ferments inulin, sucrose and raffinose with the aid of a related, but different enzyme, inulinase.

Conditions of growth were established which favor inulinase synthesis. Highest yields were obtained with inulin as the carbon source, and somewhat lower yields with raffinose. Glucose, fructose and sucrose were poor inducers of inulinase. The pH of the medium during growth on inulin had to be in the range where inulinase could act, otherwise growth was tardy and poor. In an inulin containing medium aeration favored enzyme production as a result of stimulation of growth. The inulinase content of the cells in a unit volume was generally greater than that in the culture medium. The intracellular inulinase could be solubilized quantitatively by autolysis. The intra- and extracellular inulinases were concentrated and purified to the same extent. Comparison of the two preparations with respect to substrate specificity, rate of inactivation by heat, pH optima with sucrose (4.2) and with inulin (5.0), and elution patterns from a column of diethylaminoethyl cellulose, indicated that the intra- and extracellular enzymes were identical.

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